Discogenic differentiation of human bone marrow mesenchymal stem cell with Adenovirus mediated glucose transporter-1 and hypoxia inducible factor-1α gene therapy

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Discogenic differentiation of human bone marrow mesenchymal stem cell with Adenovirus mediated glucose transporter-1 and hypoxia inducible factor-1α gene therapy

Directed by Professor Hwan-Mo Lee

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Byung Ho Lee

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This certifies that the Doctoral Dissertation of Byung Ho Lee is approved.

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The Graduate School
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On looking back on starting the process of graduate school, it was my pledge itself which made me not to spend the 3 years of the military service in vain. Without help and instruction of professor Hwan-Mo Lee and Seong-Hwan Moon, I think it would have been impossible.

For all the first five years, to start the Master's degree and finish the Doctorate degree in orthopedic surgery, I would like to thank professor Hwan-Mo Lee and Seong-Hwan Moon for dedicated supervision and guidance in both theoretical knowledge and surgical skill of the spine surgery.

As a mentor and senior, especially when I was hesitating to decide what to do in the nearest future, professors Moon always advised and lead me the best way of the situation.

I'll start my new career in Pittsburgh from this August, and I hope that the devoted efforts into this paper will act on me as a foundation to yield precious fruits.

I feel very sorry and indebt for you. My lovely wife, Mi hyun, who always supports me even with a small salary, and my

precious son and daughter, Myung hyun, and Seo yul, who always wait for the weekend with a craned neck to play with dad, because dad usually comes home late only after they fall asleep.

Byung Ho Lee

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ABSTRACT

Discogenic differentiation of human bone marrow mesenchymal stem cell with Adenovirus mediated glucose transporter-1 and hypoxia inducible factor-1α gene therapy

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(Directed by Professor Hwan-Mo Lee)

With aging and degeneration, intervertebral discs (IVDs) undergo profound and substantial changes in morphology and biochemical composition. An ideal solution for managing disc degeneration would be to repair the IVDs, by producing discogenic matrix. Recently, various approaches to biological repair of the disc function are under investigation, which are gene therapy, growth factor injection, cell therapy and cell-based tissue engineering.

Hypoxia inducible factor-1α (HIF-1α) is the key molecules regulating energy metabolism and survival in the nucleus pulposus (NP) cell. The expression of HIF was demonstrated in the NP cell of normal human IVDs and can be used as a phenotypic marker of NP cell. Hypoxia responsive glucose transporter-

1(GLUT-1) is a facilitative glucose transporter in the NP cell and also can be used as a phenotypic marker of NP cell along with HIF-1α

The discogenic induction from human bone marrow mesenchymal stem cell(BMSC) using adenoviral transduction of NP specific phenotypic factors of HIF-1α and GLUT-1 was investigated via in vitro and in vivo experiments.

The BMSC was obtained from patients during surgery for lumbar spinal stenosis. Each control and experimental groups, in vitro and in vivo experiments were performed simultaneously. In vivo study, total of 6 conditions including 1 positive control with NP cell and 1 negative control with BMSC alone were set by the combination of control, viral vector and transducted genes. All groups were consisted of BMSC, BMSC with Ad-mock, the NP cell, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 in order. The gene transduction using adenovirus and subsequent differentiation of BMSC into NP cell was confirmed by reverse-transcription polymerase chain reaction(RT-PCR) and histologic analyses. In vivo experiment, xenograft of alginate-BMSC complex on the back of mice (DVA/1J) was done. Mice were sacrificed at 2 weeks and

4 weeks after subcutaneous implantation of alginate-BMSC complexes. Analyses of each specimen including histologic test were done. The mRNA of matrix component including aggrecan and collagen type II in the differentiated NP cell was tested.

In vitro result at 24 and 48 hours culture, the BMSC with Ad-HIF-1α or Ad-GLUT-1 or both with Ad-HIF-1α and Ad-GLUT-1 groups showed increased mRNA expression of GLUT-1, aggrecan, and type II collagen compared to control and the expression level was comparable level of NP cell group. In HIF-1α mRNA expression, BMSC with Ad-HIF-1α, and the BMSC with Ad-HIF-1α and Ad-GLUT-1 groups showed increased level of expression of HIF-1α mRNA compared to those of other groups.

Based on the different oxygen conditioned culture for 96 hours, expression of HIF-1α and GLUT-1 mRNA of normoxic-hypoxic group showed analogous pattern with that of continuous normoxic group. In matrix component mRNA expression, expression pattern of aggrecan and collagen type II mRNA was comparable with that of continuous hypoxic group.

For in vivo study, the BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and

BMSC with Ad-HIF-1α and Ad-GLUT-1 groups showed positive stains for collagen type II and aggrecan, which were analogous to the NP cell group.

In conclusion, transduction of each gene of the HIF-1α, GLUT-1 using adenovirus vector was proved to be effective to induce the differentiation of BMSC into discogenic phenotype in vitro and in vivo.

Key words : intervertebral disc, bone marrow derived mesenchymal stem cell, adenovirus, HIF-1 α, GLUT-1

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I. INTRODUCTION

Low back pain affects up to 85% of people at some point during their lives, resulting in healthcare and related costs in the United States of $100 billion every year.[1-3] Numerous studies have established a causal association between degeneration of the intervertebral discs (IVDs) and low back pain.[4-6] Although disc degeneration is not commonly present until adulthood,[7] Changes to the cellular microenvironment of the IVDs begin within just a few years of birth.[8] With aging and degeneration, IVDs undergo profound substantial, morphological and cellular change.[9, 10] An ideal solution to managing IVDs

degeneration would be to repair the IVDs, producing a matrix with comparable or improved biological and biomechanical properties compared with the original.[11] Recently, various approaches to biological repair and regeneration of the IVDs function are under investigation, including gene therapy,[12,13] growth factor injection,[14] cell therapy and cell-based tissue engineering.[11,15]

Bone marrow derived mesenchymal stem cells (BMSC) have several theoretical and practical advantage over fully differentiated cells for cell therapy and tissue engineering applications.[16] Alternatively, cell populations within the IVD can be manipulated through gene therapy approaches, which involve the delivery of genes into cells through viral-vector-mediated gene transfer.[17]

Hypoxia inducible factor-1 α (HIF-1 α) is the key molecules regulating energy metabolism and survival in the nucleus pulposus (NP).[18] The expression of HIF was demonstrated in the NP cell of normal human IVDs and can be used as a phenotypic marker of NP cell.[19,20]

Glucose is an essential substrate for NP cell; it is needed both for glycolytic

metabolism and the synthesis of glycoproteins and glycosaminoglycan, which are the key molecules in preserving the gelatinous nature of the NP. An integral part of the metabolic adaptation to hypoxia is activation of genes involved in promoting anaerobic glycolysis using hypoxia responsive glucose transporter-1(GLUT-1) facilitative glucose transport [21] and also can be used as a phenotypic marker of NP cell along with HIF-1α.[20]

In the present study, we hypothesize that the transduction of those NP cell specific HIF-1α and GLUT-1 gene via adenoviral vector would induce the differentiation of BMSC into the NP cell. The potentiality and efficiency in differentiation of BMSC into the NP cell via transduction of phenotype-specific HIF-1 α and GLUT-1 gene were investigated both in vivo and in vitro.

II. MATERIALS AND METHODS

The BMSCs were obtained from patients during surgery for lumbar spinal stenosis. Reflecting control and experimental group, in vitro and in vivo experiments were performed simultaneously. In vivo study, total of 6 conditions including 1 positive control and 1 negative control were set by the combination of control, viral vector and transducted genes. All groups were consisted of BMSC, BMSC with Ad-mock, the NP cell, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 in order. Schematic diagram of the study design is shown in Figure 1.

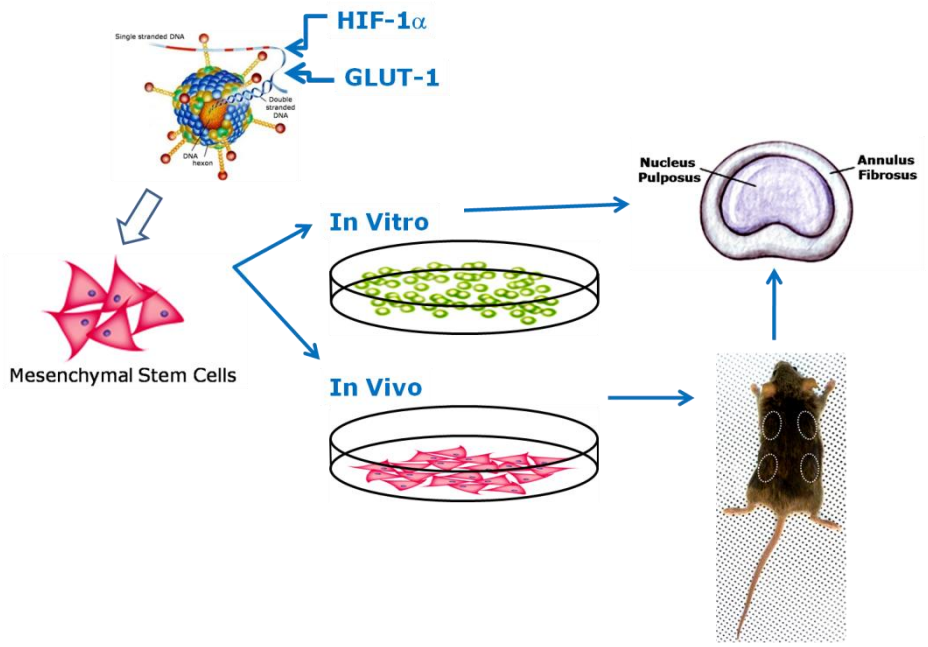


Figure 1. Schematic diagram of study design.

Both in vitro and in vivo experiments were executed simultaneously using adenoviral transduction of each HIF-1α and GLUT-1 gene alone or in combination into the BMSC. All groups were consisted of BMSC, BMSC with Ad-mock, the NP cell, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 in order. Gene-transducted BMSCs were cultured in vitro in hypoxic and normoxic conditions for 24 hours ,48 hours and 96 hours. In vivo experiments, DVI/1J mice of 4 week-old were used for subcutaneous implantation of alginate and gene transducted BMSC complex.

The gene transduction using adenovirus and subsequent differentiation of BMSCs into NP cells were confirmed by several studies such as reverse-transcription polymerase chain reaction (RT-PCR), western blot and histologic analyses. The mRNA of matrix component including aggrecan and collagen type II in the differentiated NP cell was tested as described below.

1. Isolation of the bone marrow mesenchymal stem cell (BMSC)

To attain human BMSC, bone marrow was obtained from iliac crest puncture during surgery for lumbar spinal stenosis. All patients gave informed consent before sugery. Local Ethical Committee approval was obtained for the use of the sample for research. BMSCs were isolated using a Ficoll-paque (GE Healthcare Bio-science AB, Uppsala, Sweden) density gradient method. These cells were cultured in a 75 cm^2 flask with minimum essential medium, a-modification, supplemented with 10% FBS, 1% v/v antibiotic-antimycotic, 25㎍/㎖ ascorbic acid (Gibco-BRL, Grand Island, NY, USA), at 37 ℃ in a humid atmosphere containing 5% CO_2. After 7 days, non-adherent cells were discarded and adherent cells were cultured in media supplemented and

medium changed every 2 days.

2. Ad-HIF-1α and Ad-GLUT-1 constructs

Two different adenoviral constructs were prepared for this study: Adenovirus HIF-1α construct (Ad-HIF-1α) expressing hypoxia inducible factor-1a gene and adenovirus GLUT-1 construct (Ad-GLUT-1) expressing human glucose transport-1 gene. Each recombinant adenoviral vector contained dicistronic expression cassette in which the multiple cloning site was followed by the EMCV-IRES (Agilent technologies, USA), which directed translation of a humanized recombinant green fluorescent protein (hrGFP) from a marine organism as a second open reading frame. The interest gene was fused to three contiguous copies of the FLAG® (Sigma, St. Louis, MO, USA) epitope. Recombinant virus was grown in transformed human embryonic kidney 293 cells and purified using manufacturing instructions (Vivapure® AdenoPACK, Sartorius stedim, Goettingen, Germany). Titers were determined by optical density at 260nm.

3. Adenoviral Transductions

At confluence, the MSC cell cultures were rinsed with D-PBS (Gibco-BRL, Grand Island, NY, USA) and exposed to 2ml of DMEM-LG containing one dose of Ad-HIF-1α and Ad-GLUT-1 with 120 multiplicity of infection (MOI). All cells were incubated in 5% CO_2 at 37° C incubator with humidity to prevent drying up during the transduction for one hour. Then culture medium was then added to each well, and the cells were further incubated in a 5% CO_2 at 37° C incubator with humidity.

4. Cell cytotoxicity and proliferation

A MTS (Invitrogen, Paisley, UK) assay for proliferation and cytotoxicity was used according to the manufacturers' protocol. In brief, 30ul of MTS reagent was added to each well of a 48-well microtitre plate with 200 μℓ of culture medium. The cells were incubated at 37 °C with 5% CO^2 for 1 h. Absorbance was measured at 405 nm with an ELISA microplate reader (Model 550, Bio-Rad).

5. Reverse-transcription polymerase chain reaction analysis for collagen type II, aggrecan, HIF-1α and GLUT-1

Total RNA was isolated from BMSC using QIAGEN RNeasy mini kit (QIAGEN, Valencia, CA, USA) following manufacturer's instructions. cDNA was prepared using Maxime RT premix kit (Intron, Korea). Total RNA (1 ㎍) was reverse transcribed in a final volume of 20㎕ using Oligo(dT) as primers. The cDNAs were amplified for collagen type II and aggrecan. Relative expression levels were calculated as a ratio to the average value of beta actin. The data was analyzed using TINA program. RT-PCR was done three times in vitro experiment, with the specimen of 24 hours and 48 hours culture under normoxic condition and specimen in the different oxygen condition after 96 hours culture; 96 hours of continuous culture under normoxia, 48 hours culture under hypoxia after preceding 48 hours culture under normoxia, and continuous 96 hours culture under hypoxia.

6. Western blot

Cell lysates were obtained from the specimen of 24 hours and 48 hours culture under normoxic condition and specimen in the different oxygen condition after 96 hours culture; 96 hours of continuous culture under normoxia, 48 hours culture under hypoxia after preceding 48 hours culture under normoxia, and continuous 96 hours culture under hypoxia. The lysates were fractionated on 8% sodium dodecyl sulfate–polyacrylamide gel using electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA) by electrotransfering. Proteins were immunoblotted with anti-HIF-1α (Cell Signaling Technology®, Danvers, MA, USA), anti-GLUT-1 (NOVUS biological®, Littleton, CO, USA) and anti-FLAG (Sigma, St. Louis, MO, USA). The proteins were visualized with an enhanced chemiluminescence detection system (West-Zol® plus, Intron Biotechnology, Korea) according to the manufacturing protocol.

7. Animal model

A. Alginate bead preparation

Alginate solution (2.0% w/v) was prepared by mixing alginic acid sodium salt from brown algae (medium viscosity, Sigma, St. Louis, MO, USA), 0.025M HEPES, and 0.15M NaCl in deionized water (pH 7.4), which was mildly heated on a hot plate to dissolve the alginate and then sterile filtered. Before cell encapsulation, the temperature of the alginate solution was equilibrated to 37℃. A BMSC cell suspension in alginate solution at a density of $5x10^6$ cells/mL, which was transduced by 120 MOI Ad- HIF-1α, Ad-GLUT-1, or PBS, was injected into a 200 mM $CaCl_2$ solution (Sigma, St. Louis, MO, USA) using a 20-gauge needle. Alginate beads, 2–3 mm in diameter (100,000 cells per bead), formed instantaneously and were incubated for 15 min in the $CaCl_2$ solution. The alginate beads were rinsed twice with PBS and once with DMEM, and then placed into 6-well plates with 0.5 mL medium/bead. BMSCs encapsulated in the alginate beads were cultured in DMEM, 10% FBS and 25□ g/ml L-ascorbic acid (Sigma, St. Louis, MO, USA) for 1day.

B. Xenograft study in mice

For implantation into DVA/1J mice, the prepared alginate beads-BMSC constructs were grafted subcutaneously into the back of 4-week-old female DVA/1J mice through separate incision. Surgery was performed via intramuscular injection with zoletil (Zoletil 50,Virbac Laboratories, France) and rompun (Rompun® injection, Korea). After implantation of alginate beads, all wounds were closed in a routine manner using Nylon 4.0 suture material. No secondary operations were performed in the sham. On 2 weeks and 4 weeks, the mice were killed for analysis and the tissue is extracted.

C. RT-PCR for collagen type 2, aggrecan and HIF-1α

Total RNA was isolated from MSC using QIAGEN RNeasy mini kit (QIAGEN) following manufacturer's instructions. cDNA was prepared using Maxime RT premix kit (Intron, Korea) according to the manufacturer's instructions. The cDNAs were amplified for collagen type 2, aggrecan and HIF-1α. Relative expression levels were calculated as a ratio to the average value of beta actin. The data was analyzed using TINA program.

D. Histological Analysis

After macroscopic evaluation, the tissues from back were individually processed for histological studies. Each tissue was fixed in 4% formaline, and 10% sodium citrate for approximately 5 minutes and processed for paraffin-embeded tissue and sectioning into sagittal sections (5 mm thick). Sections were stained with H & E(Sigma, St. Louis, MO, USA), collagen type 2 (Chondris, Inc., Redmond, WA, USA), aggrecan (abcam®, cambridge, UK), STRO (R&D systems, Inc., Minneapolis, MN, USA), for mineralization and alcian blue (Sigma, St. Louis, MO, USA) for evaluation.

III. RESULTS

1. In vitro

A. Cell cytotoxicity

In cell cytotoxicity analysis, each group except the BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed no significant difference. The BMSC with Ad-HIF-1α and Ad-GLUT-1 showed highest cytotoxicity due to double dose of transducted adenovirus (240 MOI: multiplicity of infection) compared to those of other groups (120 MOI). (Figure 2)

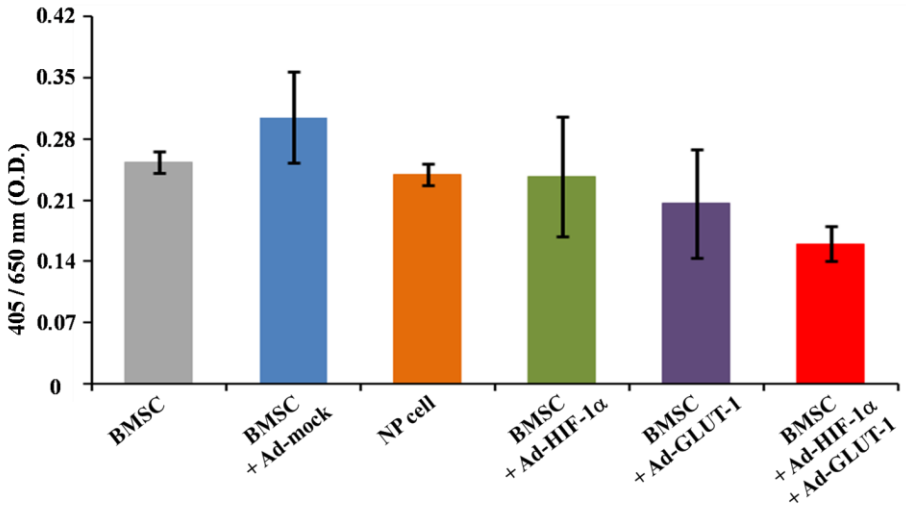


Figure 2. Cell cytotoxicity in vitro.

In cell cytotoxicity analysis, each group showed no significant difference between them except BMSC with Ad-HIF-1α and Ad-GLUT-1. BMSC with Ad-HIF-1α and Ad-GLUT-1 showed highest cytotoxicity due to double dose of transducted adenovirus (240 MOI: multiplicity of infection) compared to those of other groups (120 MOI). Because of highest transducted viral dose, BMSC with Ad-HIF-1α and Ad-GLUT-1 showed the resultant lowest cell counts in all groups. MOI : multiplicity of infection (viral titer/cell number)

B. Cell proliferation

In cell proliferation analysis, all of the BMSC groups showed comparable results at 7 days. NP cell group showed the highest cell proliferation at 7 days, which is shown in Figure 3.

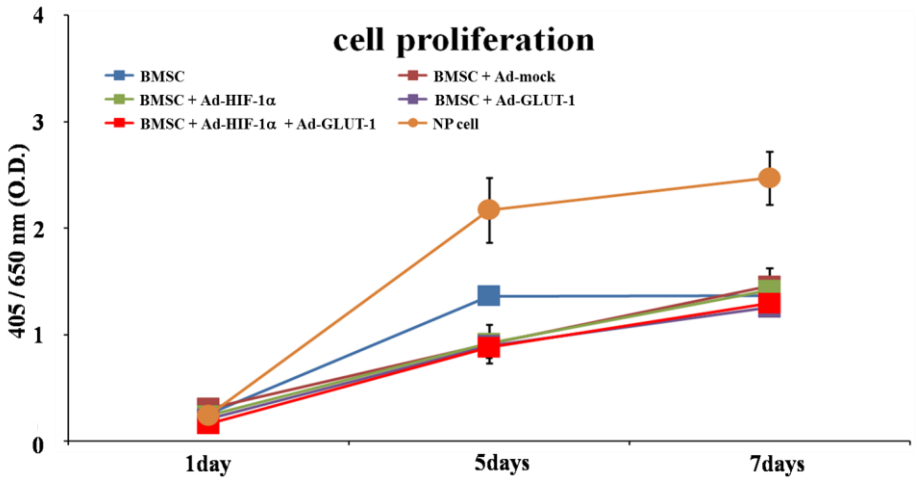


Figure.3 Cell proliferation in vitro.

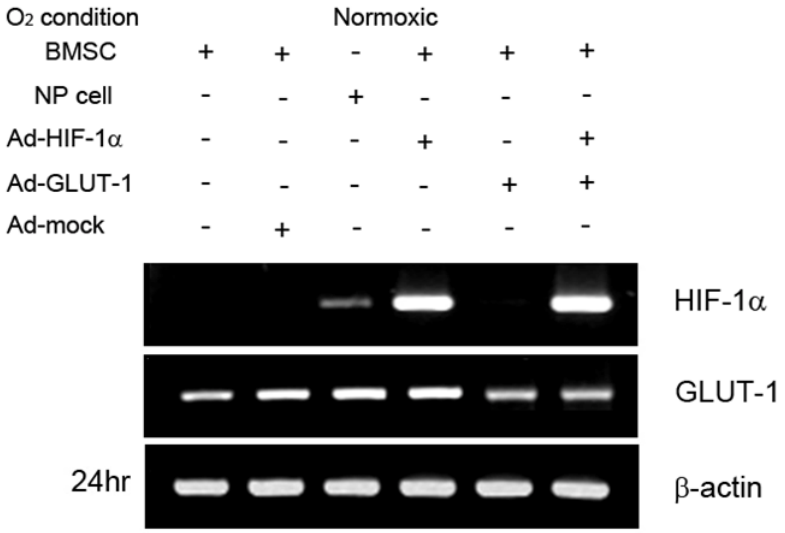
NP cell group showed the highest cell proliferation compared to other BMSC groups. Other groups showed comparable cellular proliferation rate at 7 days.

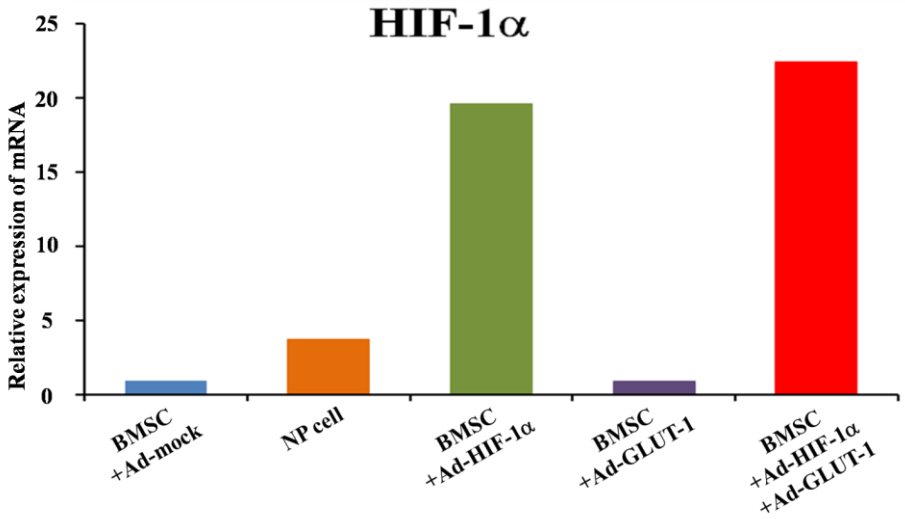
C . RT-PCR of HIF-1α and GLUT-1

(A) Normoxic condition at 24 and 48 hours

In normoxic condition at 24 hours, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed almost 19 fold and 22 fold increase in HIF-1α mRNA expression compared to that of BMSC. Also NP cell group showed 3 fold increase in HIF-1α mRNA expression.

All groups revealed no significantly different level of mRNA expression of GLUT-1. It ranged from 5% to 40% increase of GLUT-1 mRNA expression compared to that of BMSC. (Figure 4)


O2 condition
Normoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
HIF-1α
GLUT-1
24hr
β-actin


HIF-1α
Relative expression of mRNA
25
20
15
10
5
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

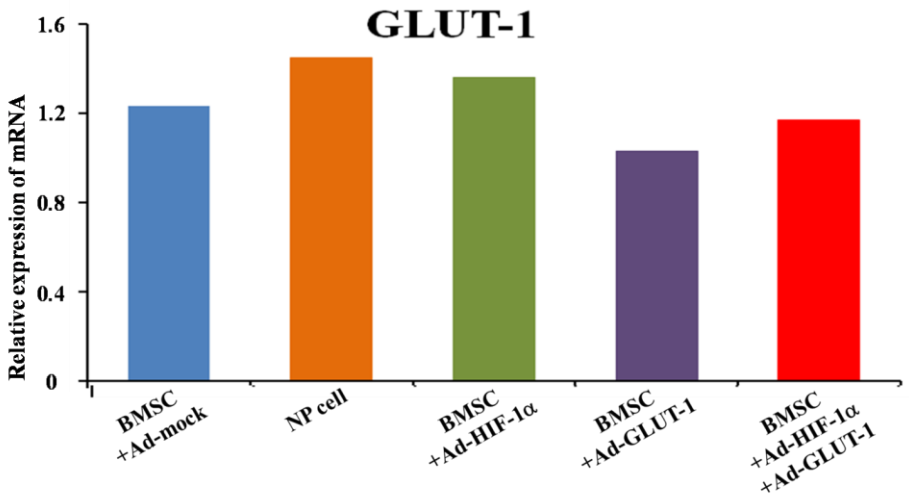
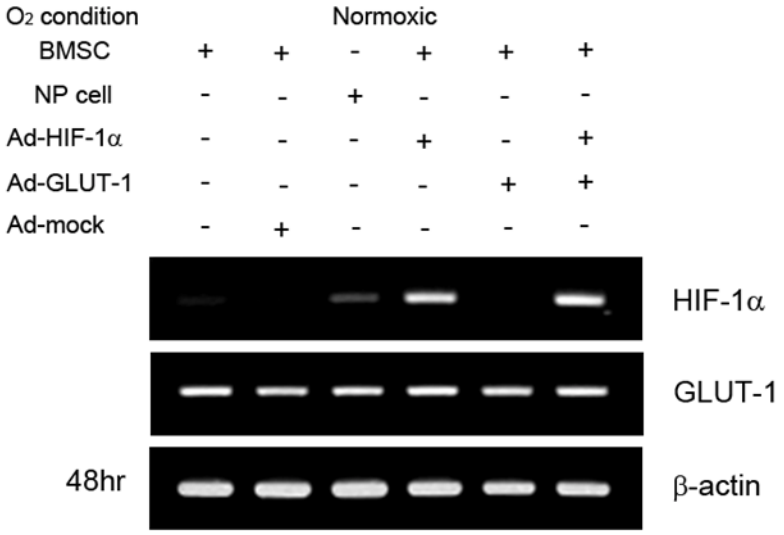
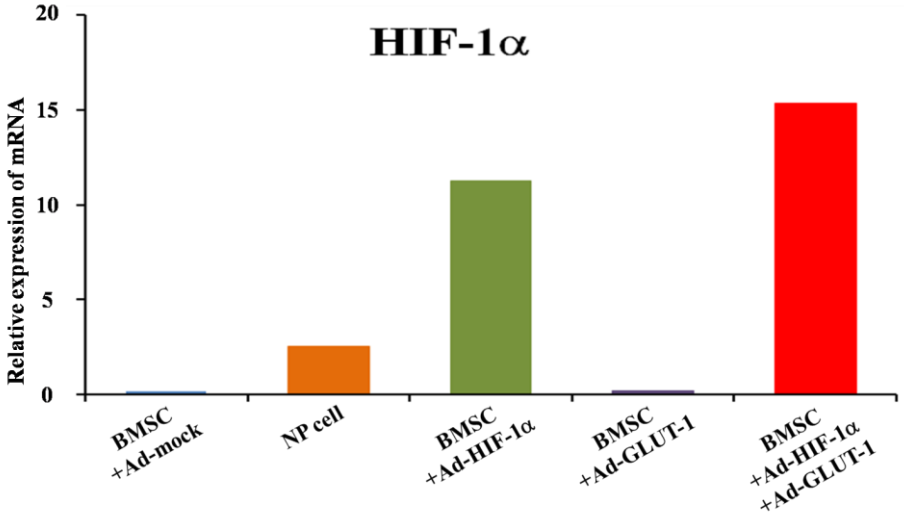


Figure 4. RT-PCR result of HIF-1α and GLUT-1 at 24 hours in vitro culture in normoxic condition.

In HIF-1α, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed almost 19 fold and 22 fold increase in HIF-1α mRNA expression compared to that of BMSC. In GLUT-1 mRNA expression, all groups showed no significantly different level of mRNA expression ranged from 5% to 40% increase of GLUT-1 mRNA expression compared to that of BMSC.

In normoxic condition at 48 hours, NP cell, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group demonstrated 2 fold, 12 fold and 15 fold increase in HIF-1α mRNA expression compared to that of BMSC. In GLUT-1 mRNA expression, BMSC with Ad-mock and NP cell group showed 20% to 30% decrease in GLUT-1 mRNA expression compared to that of BMSC. Contrary to those, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 revealed 10% and 15% increase in GLUT-1 mRNA expression compared to that of BMSC. BMSC with Ad-HIF-1α showed comparable level of GLUT-1 mRNA expression with BMSC. (Figure 5)


O2 condition
Normoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
HIF-1α
GLUT-1
48hr
β-actin


HIF-1α
Relative expression of mRNA
20
15
10
5
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

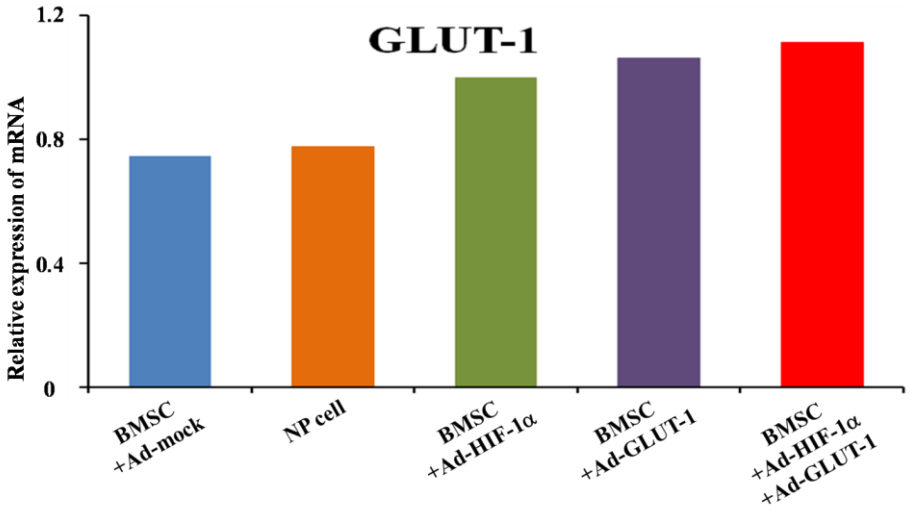


Figure 5. RT-PCR result of HIF-1α and GLUT-1 at 48 hours in vitro culture in normoxic condition.

BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 12 fold and 15 fold increase in HIF-1α mRNA expression compared to that of BMSC.

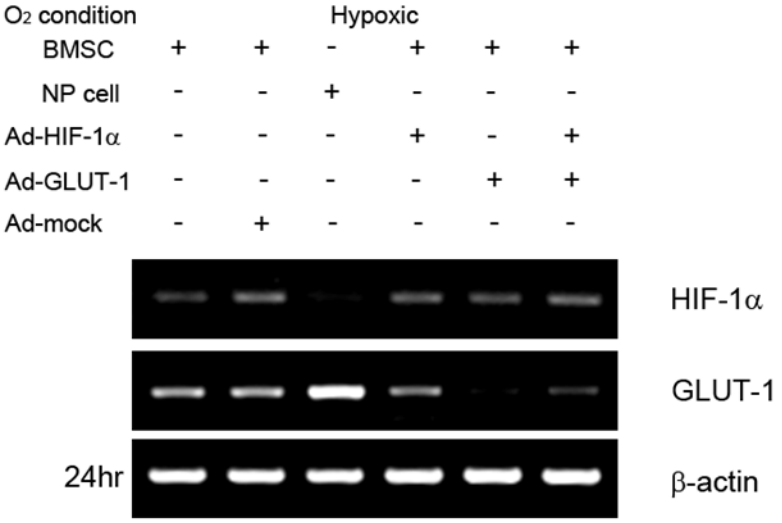
In GLUT-1 mRNA expression, BMSC with Ad-mock and NP cell group demonstrated 20% to 30% decrease but BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 revealed 10% and 15% increase in GLUT-1 mRNA expression compared to that of BMSC.

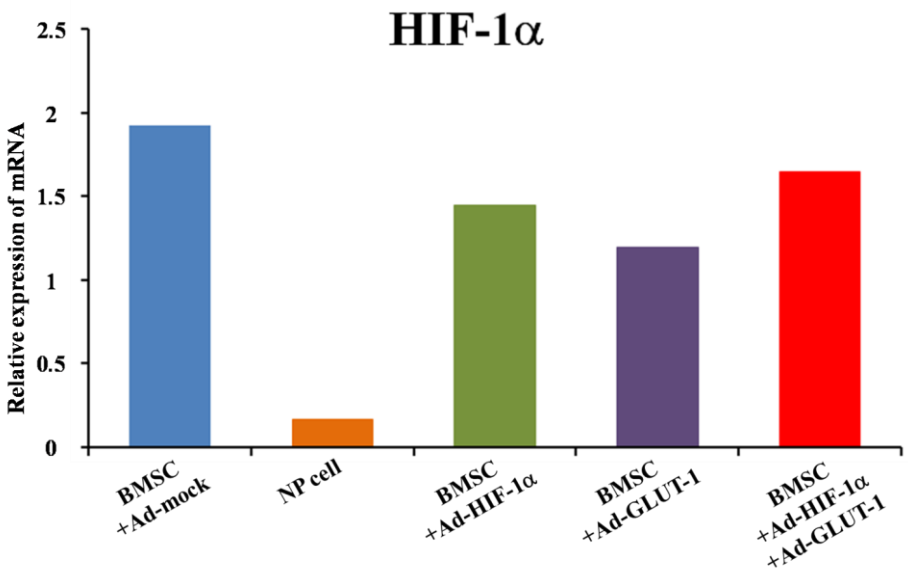
(B) Hypoxic conditions at 24 and 48 hours

In vitro culture results for 24 hours and 48 hours in hypoxic condition were different from the results in normoxic condition.

In hypoxic condition at 24 hours, BMSC with Ad-mock, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed each of 90%, 45%, 25% and 70% increase in HIF-1α mRNA expression compared to that of BMSC. But, NP cell group demonstrated 80% decrease in HIF-1α mRNA expression compared to that of BMSC.

Only NP cell group demonstrated 120% increase in mRNA expression of GLUT-1 compared to that of BMSC. BMSC with Ad-mock demonstrated comparable level of GLUT-1 mRNA expression. BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group revealed 40%, 90% and 80% decrease in GLUT-1 mRNA expression. These results of HIF-1α and GLUT-1 mRNA expression are shown in Figure 6.


O2 condition
Hypoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
HIF-1α
GLUT-1
24hr
β-actin


HIF-1α
Relative expression of mRNA
2.5
2
1.5
1
0.5
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

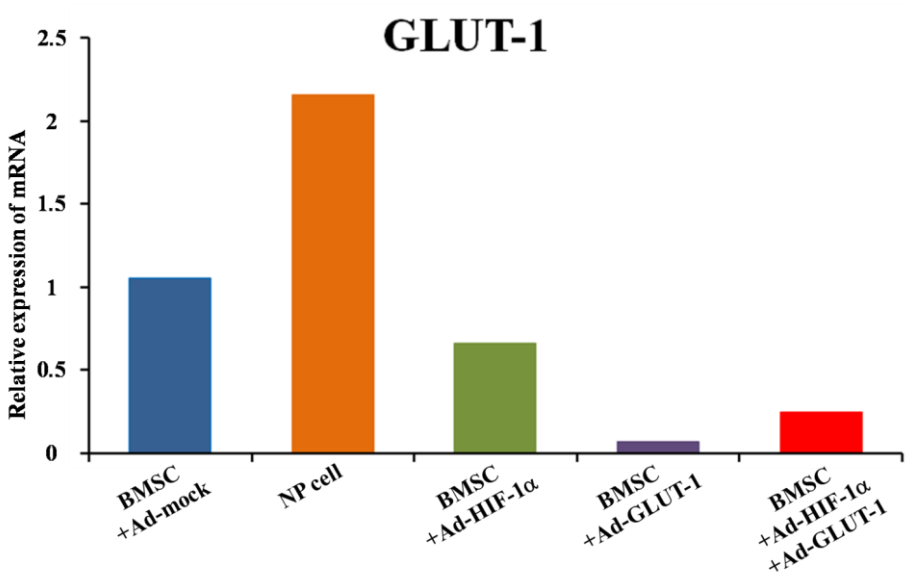
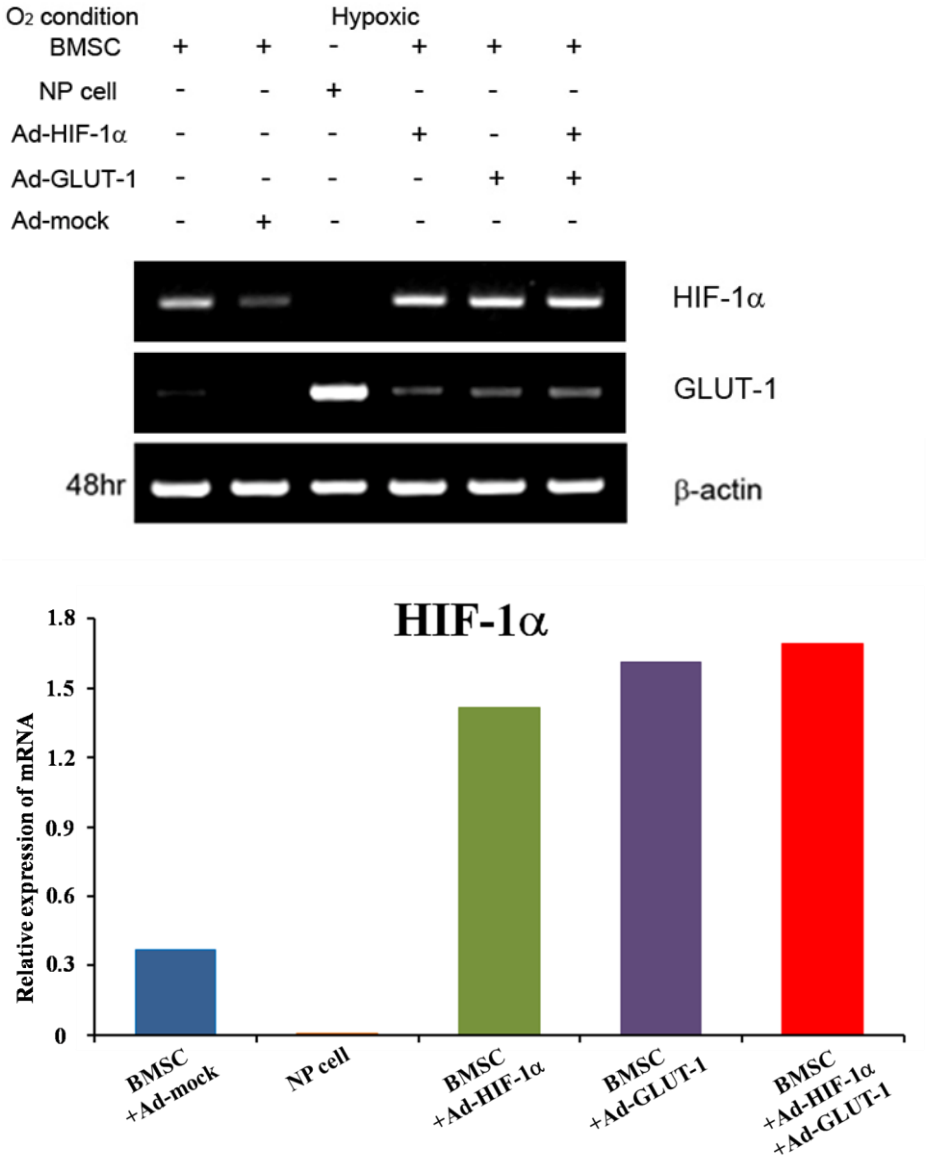


Figure 6. RT-PCR result of HIF-1α and GLUT-1 at 24 hours in vitro culture in hypoxic condition.

BMSC with Ad-mock, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed each of 90%, 45%, 25% and 70% increase in HIF-1α mRNA expression compared to that of BMSC. But in the expression of GLUT-1 mRNA, only NP cell group showed 120% increase in mRNA expression of GLUT-1 compared to that of BMSC.

In vitro culture results for 48 hours in hypoxic condition were different from those of in vitro result for 24 hours. (Figure 7) In HIF-1α mRNA expression, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 groups showed each of 30%, 60% and 70% increase compared to that of BMSC. But, the BMSC with Ad-mock demonstrated 60% decrease in HIF-1α mRNA expression compared to that of BMSC. NP group showed no expression of HIF-1α mRNA in this condition.

In GLUT-1 mRNA expression, NP cell group, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 groups showed 37 fold, 4 fold, 6 fold, and 8 fold increase in mRNA expression of GLUT-1 compared to that of BMSC. BMSC with Ad-mock demonstrated no GLUT-1 mRNA expression.


O2 condition
Hypoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
HIF-1α
GLUT-1
48hr
β-actin
HIF-1α
Relative expression of mRNA
1.8
1.5
1.2
0.9
0.6
0.3
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

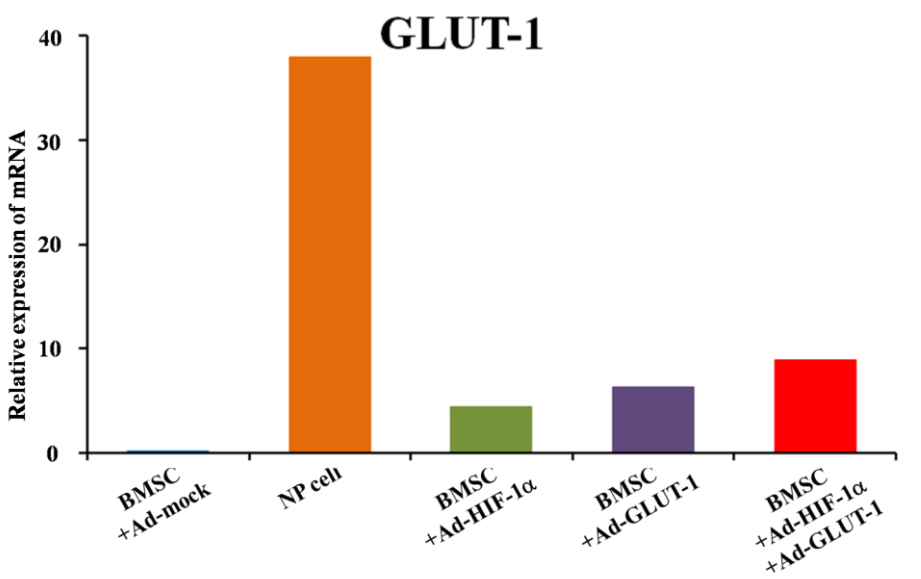


Figure 7. RT-PCR result of HIF-1α and GLUT-1 at 48 hours in vitro culture in hypoxic condition.

In HIF-1α mRNA expression, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 groups showed each of 30%, 60% and 70% increase in HIF-1α mRNA expression compared to that of BMSC. In GLUT-1 mRNA expression, NP cell group, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 groups showed 37 fold, 4 fold, 6 fold, and 8 fold increase in mRNA expression of GLUT-1 compared to that of BMSC.

(C) Different oxygen conditions at 96 hours

In expression of HIF-1α and GLUT-1 mRNA, normoxic-hypoxic condition at 96 hours demonstrated analogous pattern with those in continuous normoxic condition at 96 hours. (Figure 8)

In continuous normoxic condition for 96 hours

In HIF-1α mRNA expression, BMSC with Ad-HIF-1α, BMSC with Ad-HIF-1α and Ad-GLUT-1, BMSC with Ad-GLUT-1 and NP cell group showed increased level of HIF-1α mRNA in order compared to that of BMSC.

In GLUT-1 mRNA expression, BMSC with Ad-GLUT-1, NP cell, BMSC with Ad-HIF-1α and Ad-GLUT-1 and BMSC with Ad-HIF-1α group showed increased level of GLUT-1 mRNA expression in order compared to that of BMSC.

In normoxic (48 hours) and hypoxic (48 hours) condition

In HIF-1α mRNA expression, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group revealed increased level of HIF-1α mRNA expression in order compared to that of BMSC.

In GLUT-1 mRNA expression, BMSC with Ad-HIF-1α and Ad-GLUT-1, BMSC with Ad-GLUT-1 and NP cell group demonstrated increased level of GLUT-1 mRNA expression in order compared to that of BMSC.

In continuous hypoxic condition for 96 hours

In HIF-1α mRNA expression, BMSC with Ad-HIF-1α and Ad-GLUT-1, and BMSC with Ad-GLUT-1 group demonstrated increased level of HIF-1α mRNA expression in order compared to that of BMSC. BMSC with Ad-mock and NP cell groups showed comparable level of HIF-1α mRNA expression with BMSC. BMSC with Ad-HIF-1α demonstrated decreased level of HIF-1α mRNA expression.

In GLUT-1 mRNA expression, BMSC with Ad-GLUT-1, BMSC with Ad-HIF-1α and Ad-GLUT-1, NP cell, BMSC with Ad-HIF-1α and BMSC with Ad-mock group showed increased level of GLUT-1 mRNA expression in order compared to that of BMSC.

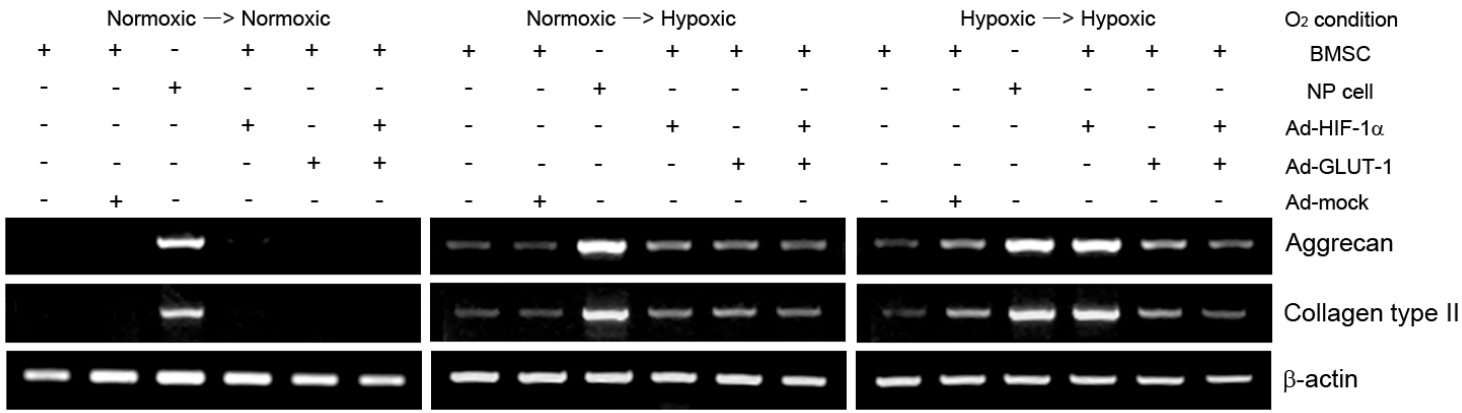


Figure 8. RT-PCR result of phenotypic expression in the different oxygen condition for 96 hours.

In HIF-1α and GLUT-1 mRNA expression, expression of HIF-1α and GLUT-1 mRNA in normoxic-hypoxic condition at 96 hours showed analogous pattern with those in continuous normoxic condition at 96 hours.

In continuous normoxic condition at 96 hours, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 revealed most increased level of HIF-1α mRNA expression in order compared to that of BMSC. In GLUT-1 mRNA expression, the BMSC with Ad-GLUT-1, NP cell and the BMSC with Ad-HIF-1α and Ad-GLUT-1 demonstrated increased level of GLUT-1 mRNA expression in order compared to that of BMSC.

In normoxic (48 hours) and hypoxic (48 hours) condition, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-

Figure 8. Continued

1 group demonstrated increased level of HIF-1α mRNA expression in order compared to that of BMSC. In GLUT-1 mRNA expression, BMSC with Ad-HIF-1α and Ad-GLUT-1, BMSC with Ad-GLUT-1 and NP cell group showed increased level of GLUT-1 mRNA expression in order compared to that of BMSC in order.

In Continuous hypoxic condition, BMSC with Ad-HIF-1α and Ad-GLUT-1, and BMSC with Ad-GLUT-1 group showed increased level of HIF-1α mRNA expression in order compared to that of BMSC. In GLUT-1 mRNA expression, BMSC with Ad-GLUT-1, BMSC with Ad-HIF-1α and Ad-GLUT-1, NP cell and BMSC with Ad-HIF-1α group showed increased level of GLUT-1 mRNA expression compared to that of BMSC.

D. Western blot

In the HIF-1α protein analysis using anti-HIF-1α antibody, only BMSC with Ad-HIF-1α group showed positive result of HIF-1α protein. In additional analysis using anti-FLAG antibody, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of synthesized HIF-1α protein by the transducted Ad-HIF-1α gene. The result for detecting the induced HIF-1α is shown in Figure 9.

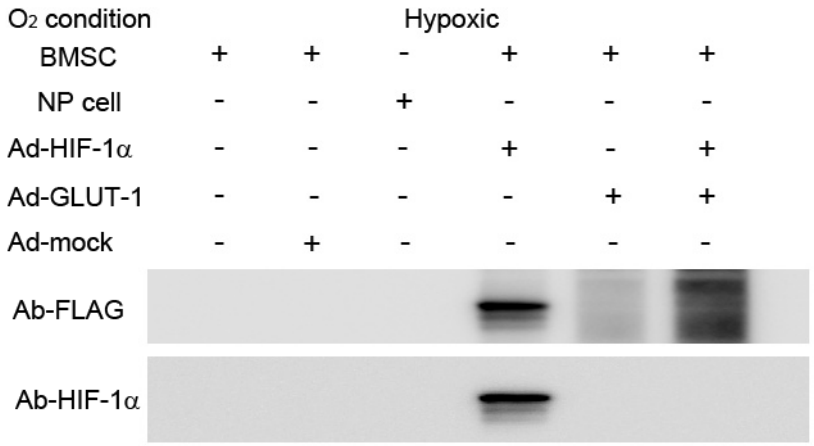


Figure 9. Immunoblotting assay for HIF-1α protein.

In the HIF-1α protein analysis using anti-HIF-1α antibody, only BMSC with Ad-HIF-1α group showed positive result of HIF-1α protein.In additional analysis using anti-FLAG antibody, BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of synthesized HIF-1α protein by transducted Ad-HIF-1α gene. It was due to relative strong detection of HIF-1α protein in BMSC with Ad-HIF-1α compared to BMSC with Ad-HIF-1α and Ad-GLUT-1. BMSC: bone marrow mesenchymal stem cell, Ad: adenovirus, HIF-1α : hypoxia inducible factor-1 alpha, GLUT-1: glucose transporter-1

In the GLUT-1 protein analysis using anti-GLUT-1 antibody, all group showed positive result of GLUT-1 protein but only BMSC with Ad-GLUT-1 group demonstrated strong positive result of GLUT-1 protein. In additional analysis using anti-FLAG antibody, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of synthesized GLUT-1 protein by the transducted Ad-GLUT-1 gene. The result for detecting the synthesized GLUT-1 protein is shown in Figure.10.

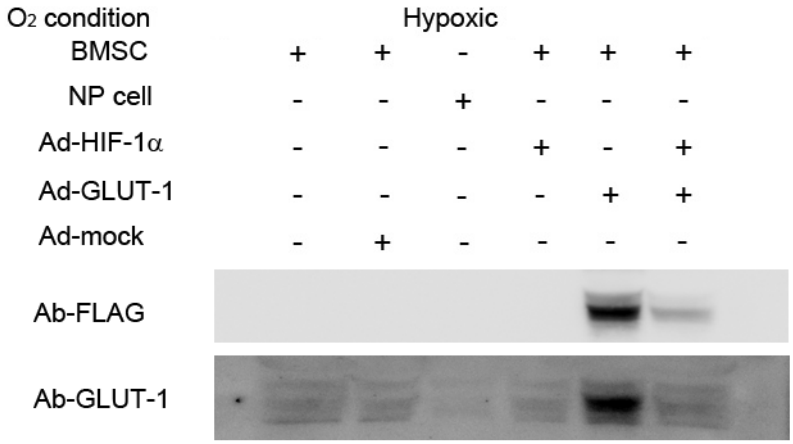


Figure. 10 Immunoblotting assay for GLUT-1 protein.

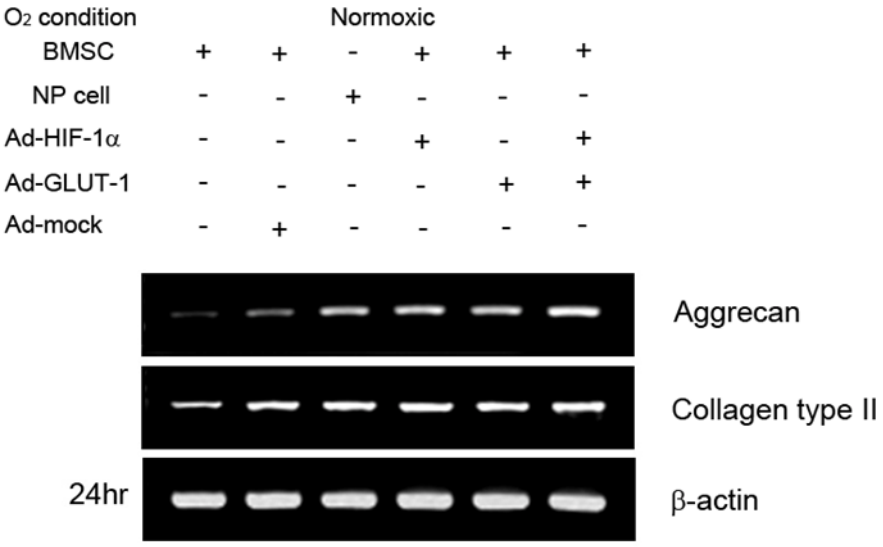
In the GLUT-1 protein analysis using anti-GLUT-1 antibody, all group showed positive result of GLUT-1 protein. In additional analysis using anti-FLAG antibody, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of synthesized GLUT-1 protein by the transducted GLUT-1 gene. BMSC: bone marrow mesenchymal stem cell, Ad: adenovirus, HIF-1α : hypoxia inducible factor-1 alpha, GLUT-1: glucose transporter-1

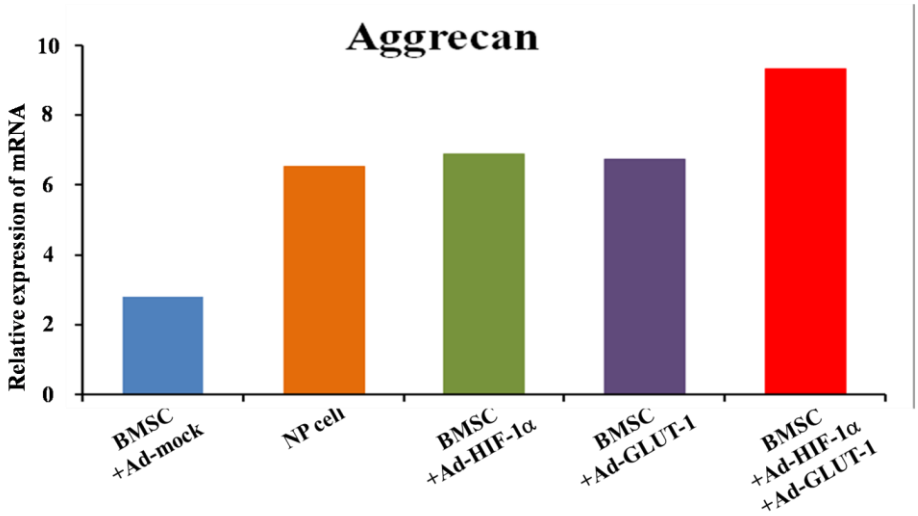
E . RT-PCR of aggrecan and collagen type II

(A) Normoxic condition at 24 and 48 hours

In aggrecan mRNA expression at 24 hours, the BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 9 fold increase of aggrecan mRNA expression compared to that of BMSC. Other groups including NP cell, BMSC with Ad-HIF-1α and BMSC with Ad-GLUT-1 showed 6 fold increases in aggrecan mRNA expression at 24 hours.

In collagen type II mRNA expressionat 24 hours, all group showed from 180% to 300% increased expression of collagen type II mRNA compared to that of BMSC. Also, the BMSC with Ad-HIF-1α and Ad-GLUT-1 group revealed the highest increase of collagen type II mRNA expression among all groups. Thest results are also shown in Figure 11.


O2 condition
Normoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
Aggrecan
Collagen type II
24hr
β-actin


Aggrecan
Relative expression of mRNA
10
8
6
4
2
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

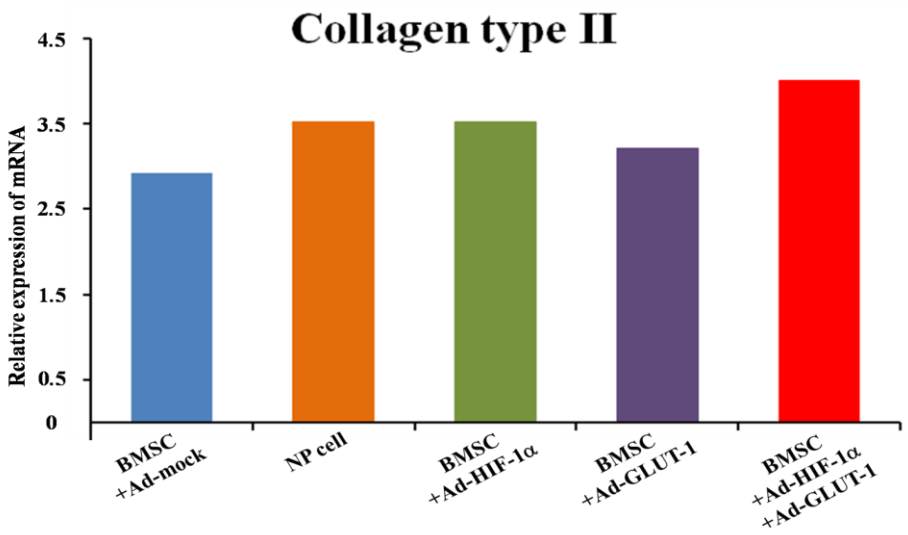
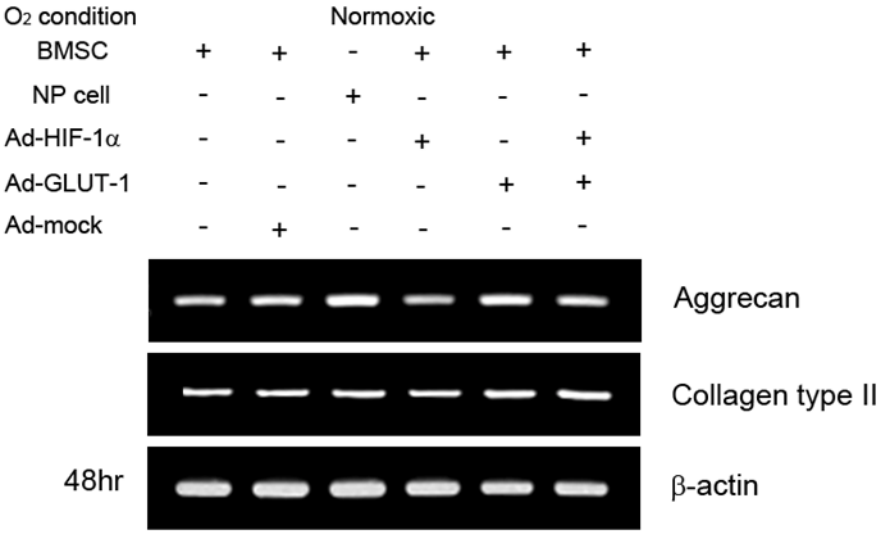


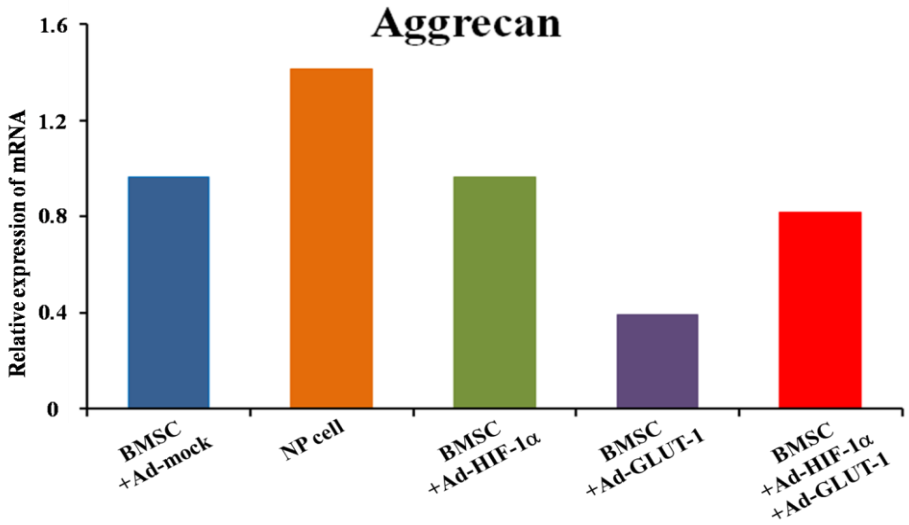
Figure 11. RT-PCR result of aggrecan and collagen type II at 24 hours in vitro culture in normoxic condition. In the aggrecan mRNA expression, BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 9 fold increase of aggrecan mRNA expression compared to that of BMSC. In collagen type II mRNA expression, all group showed from 180% to 300% increased expression of collagen type II mRNA compared to that of BMSC..

BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed highest increase in aggrecan and collagen type II mRNA expression in all groups.

In the aggrecan mRNA expression at 48 hours, NP cell showed 40% increase in aggrecan mRNA expression compared to that of BMSC. Other groups including BMSC with Ad-mock and BMSC with Ad-HIF-1α showed 10% decreases in aggrecan mRNA expression compared to that of BMSC.

In collagen type II mRNA expression at 48 hours, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 60% and 65% increase in expression of collagen type II mRNA compared to that of BMSC. Also, other groups showed comparable level of collagen type II mRNA expression as much as that of BMSC. (Figure 12)


O2 condition
Normoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
Aggrecan
Collagen type II
48hr
β-actin


Aggrecan
Relative expression of mRNA
1.6
1.2
0.8
0.4
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

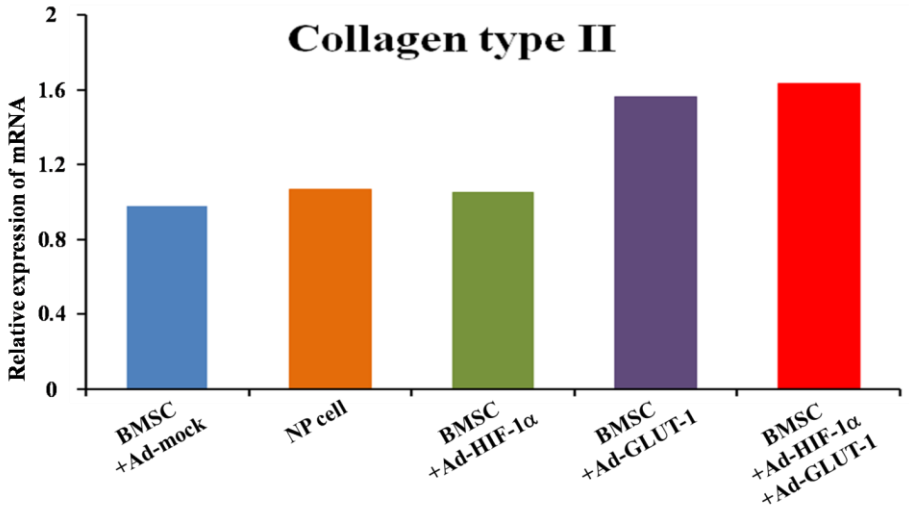


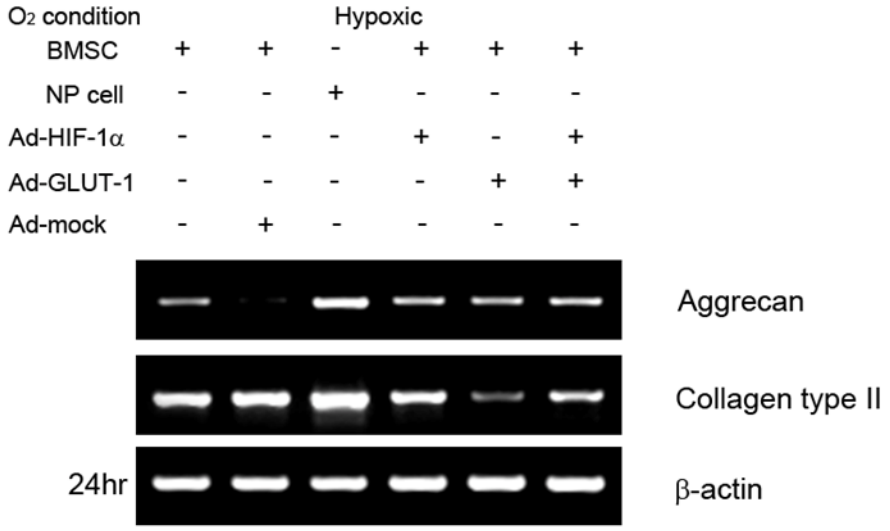
Figure 12. RT-PCR result of aggrecan and collagen type II at 48 hours in vitro culture in normoxic condition.

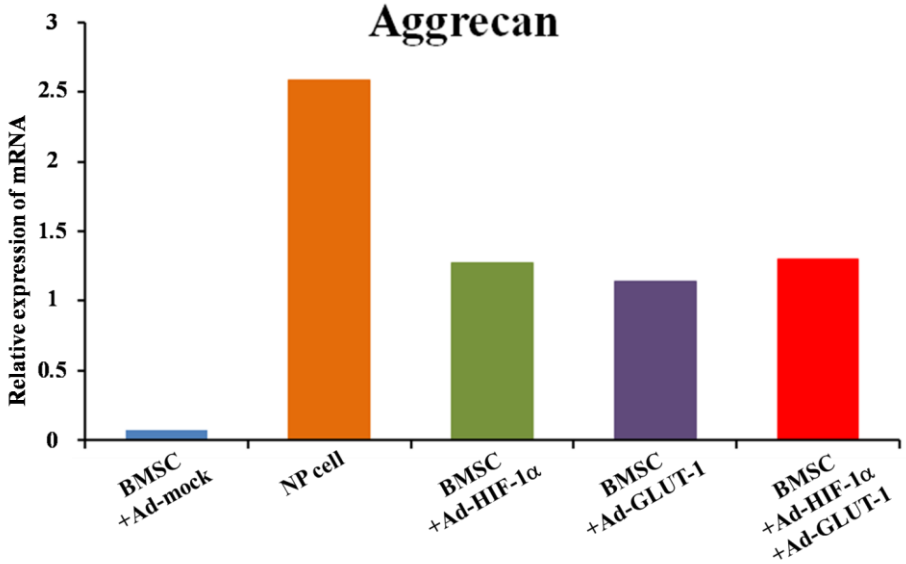
In the aggrecan mRNA expression, NP cell group showed 40% increase in aggrecan mRNA expression compared to that of BMSC. In collagen type II mRNA expression, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 60% and 65% increase in expression of collagen type II mRNA compared to that of BMSC.

(B) Hypoxic conditions at 24 and 48 hours

In the aggrecan mRNA expression at 24 hours, NP cell group demonstrated 160% increase in aggrecan mRNA expression compared to that of BMSC. BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 20%, 10% and 20% increased level of aggrecan mRNA expression compared to that of BMSC. BMSC with Ad-mock showed 90% decreased level of aggrecan mRNA.

In collagen type II mRNA expression at 24 hours, NP cell group showed 45% increase in collagen type II mRNA expression compared to that of BMSC. BMSC with Ad-mock group showed comparable level of collagen type II mRNA expression with BMSC. BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 30%, 80% and 50% decreased expression of collagen type II mRNA expression. (Figure 13)


O2 condition
Hypoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
Aggrecan
Collagen type II
24hr
β-actin


Aggrecan
Relative expression of mRNA
3
2.5
2
1.5
1
0.5
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

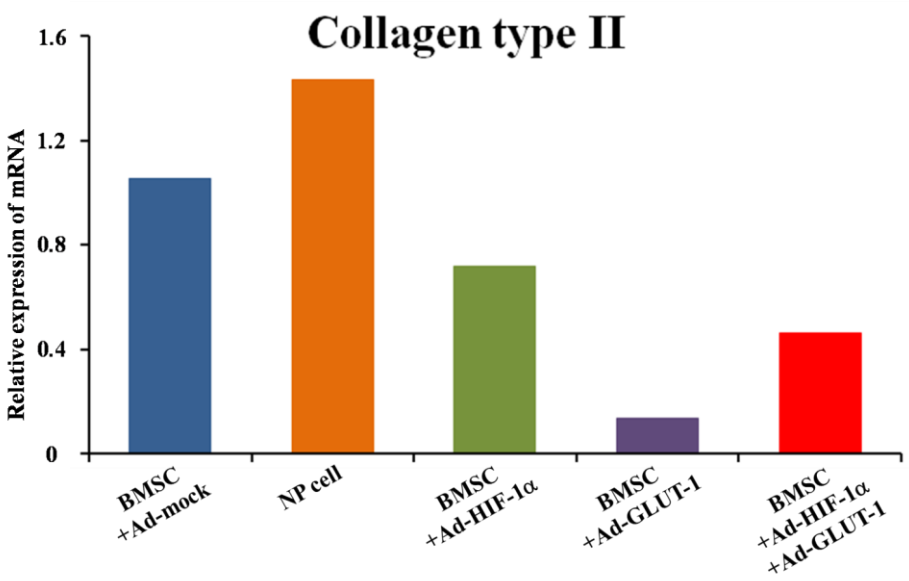
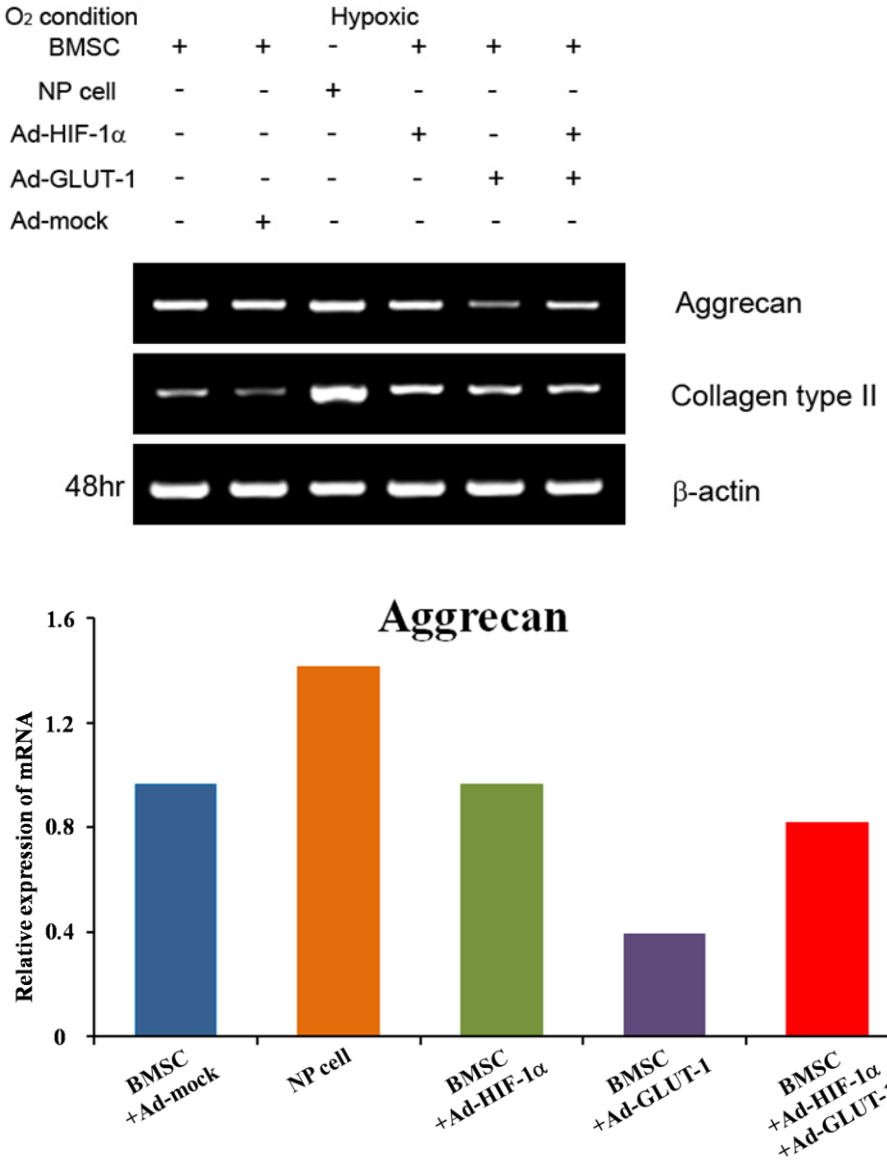


Figure 13. RT-PCR result of aggrecan and collagen type II at 24 hours in vitro culture in hypoxic condition.

NP cell group demonstrated 160% increase in aggrecan mRNA expression compared to that of BMSC. In collagen type II mRNA expression, NP cell group showed 45% increase in collagen type II mRNA expression compared to that of BMSC.

In aggrecan mRNA expression at 48 hours, NP cell group demonstrated 40% increase compared to that of BMSC. BMSC with Ad-mock and BMSC with Ad-HIF-1α demonstrated analogous level of aggrecan mRNA expression compared to that of BMSC. BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 60% and 20% decreased level of aggrecan mRNA expression compared to that of BMSC.

In collagen type II mRNA expression at 48 hours, NP cell demonstrated 4.5 fold increase in collagen type II mRNA expression compared to that of BMSC. BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed 100%, 80% and 90% increased expression of collagen type II mRNA expression. BMSC with Ad-mock group showed 40% decrease of collagen type II mRNA expression. (Figure 14)


O2 condition
Hypoxic
BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
48hr
Aggrecan
Collagen type II
β-actin
Aggrecan
Relative expression of mRNA
1.6
1.2
0.8
0.4
0
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

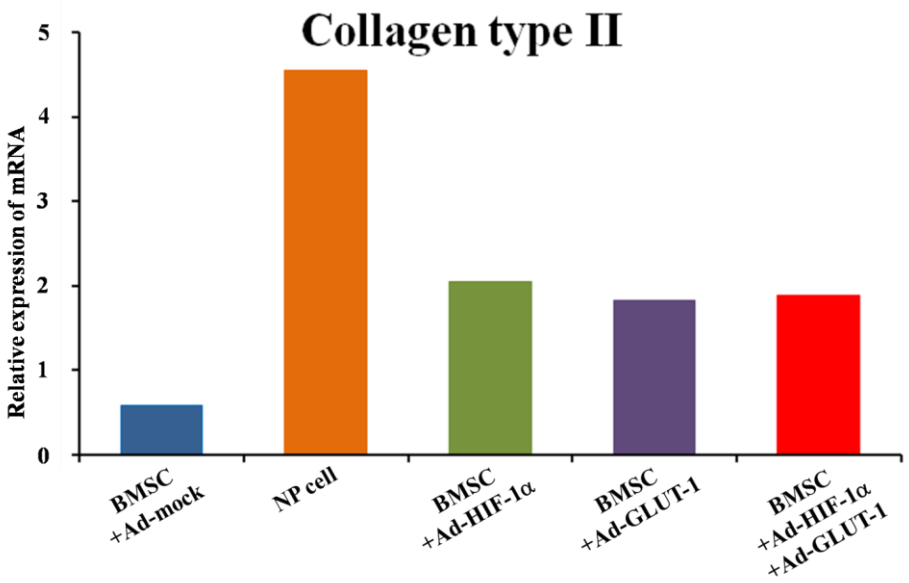


Figure 14. RT-PCR result of aggrecan and collagen type II at 48 hours in vitro culture in hypoxic condition.

In the aggrecan mRNA expression, NP cell group demonstrated 40% increase compared to that of BMSC. In collagen type II mRNA expression, NP cell showed 4.5 fold increase in collagen type II mRNA expression compared to that of BMSC.

(C) Different oxygen conditions at 96 hours

In matrix component mRNA expression, expression of aggrecan and collagen type II mRNA in continuous normoxic condition at 96 hours demonstrated analogous patterns with those in continuous hypoxic group. (Figure 15)

In continuous normoxic condition for 96 hours

In aggrecan and collagen type II mRNA expression, only NP cell group revealed increased level of aggrecan and collagen type II mRNA expression compared to that of BMSC.

In normoxic (48 hours) and hypoxic (48 hours) condition

In aggrecan mRNA expression, only NP cell group revealed significantly increased level of aggrecan mRNA expression compared to that of BMSC. Also, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 showed increased expression of aggrecan mRNA compared to that of BMSC.

In collagen type II mRNA expression, only NP cell group showed

significantly increased level of collagen type II mRNA expression compared to that of BMSC. Also, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 showed increased expression of collagen type II mRNA compared to that of BMSC.

In continuous hypoxic condition for 96 hours

In aggrecan mRNA expression, NP cell, BMSC with Ad-HIF-1α, and BMSC with Ad-GLUT-1, BMSC with Ad-mock and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed significantly increased level of aggrecan mRNA expression in order compared to that of BMSC.

In collagen type II mRNA expression, NP cell ,the BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1, BMSC with Ad-mock and BMSC with Ad-HIF-1α and Ad-GLUT-1 group revealed significantly increased level of collagen type II mRNA expression in order compared to that of BMSC.

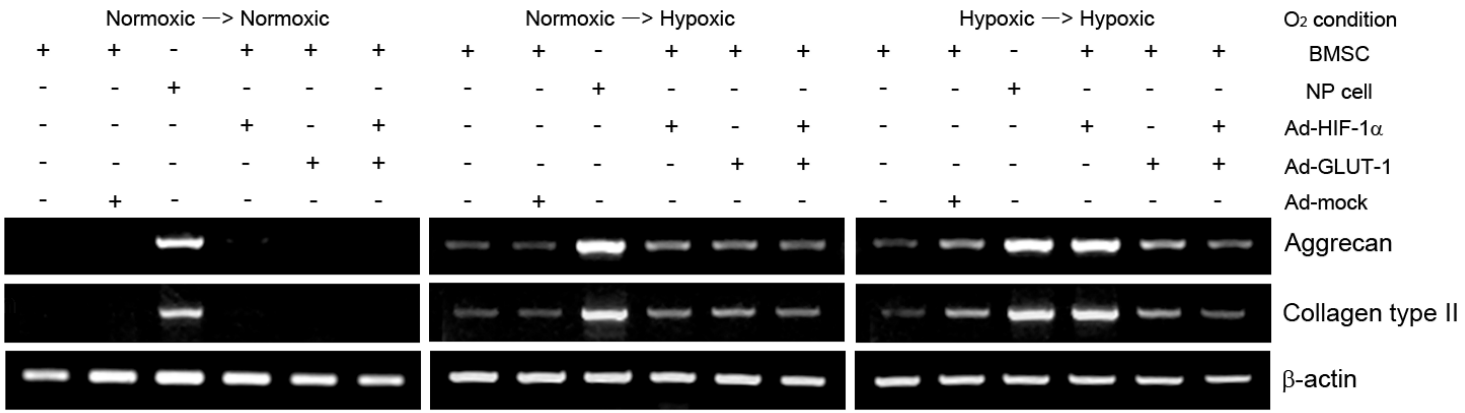


Figure 15. RT-PCR result of matrix synthesis in the different oxygen condition for 96 hours.

In matrix component mRNA, expression of aggrecan and collagen type II mRNA in continuous normoxic condition at 96 hours demonstrated analogous patterns with those in continuous hypoxic group.

In continuous normoxic condition at 96 hours, only NP cell group showed significantly increased level of aggrecan and collagen type II mRNA expression compared to that of BMSC.

In normoxic (48 hours) and hypoxic (48 hours) condition, only NP cell group showed significantly increased level of aggrecan and collagen type II mRNA expression compared to that of BMSC.

In continuous hypoxic condition, NP cell and BMSC with Ad-HIF-1α group showed significantly increased level of aggrecan and collagen type II mRNA expression in order compared to that of BMSC.

2. In vivo

In vivo study was perfomred according to the time table shown in Table 1. Each of result was described below.

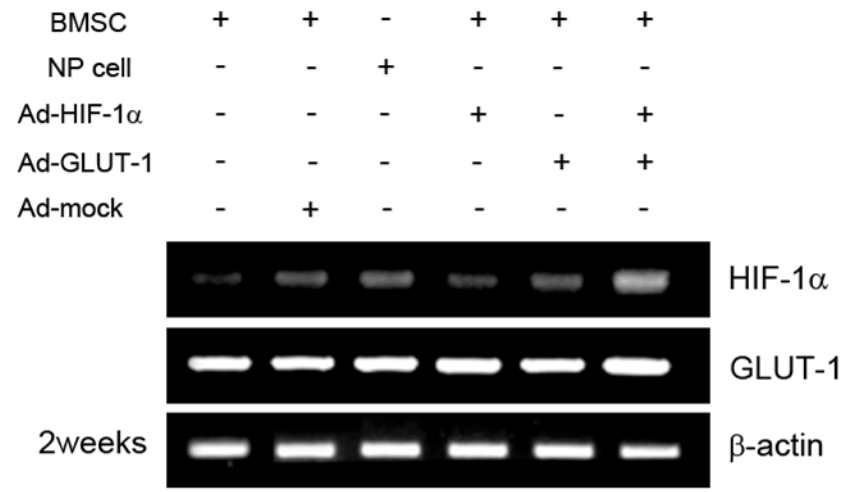
Table 1. Summary of group and time-table of in vivo experiment

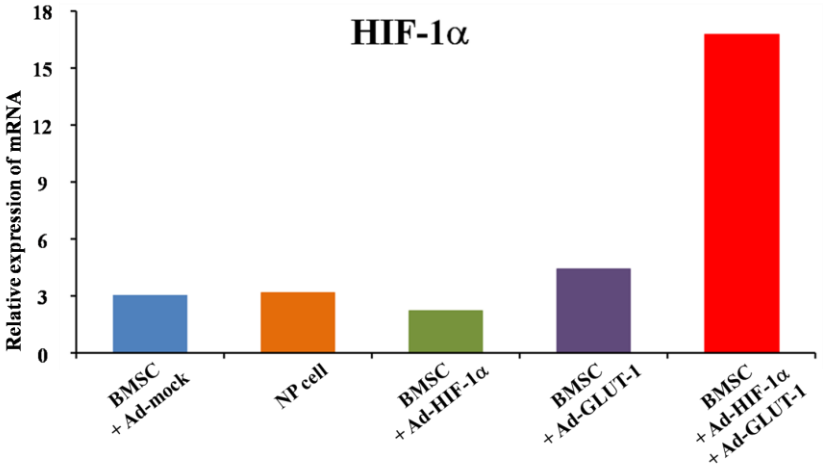
Time	***Control-1 (BMSC) N=7***	***Control-2 (BMSC with Ad-mock) N=7***	***Control-3 (Nucleus pulposus) N=7***	***BMSC with Ad -HIF-1a N=7***	***BMSC with Ad -GLUT-1 N=7***	***BMSC With Ad -HIF-1a and Ad-GLUT-1 N=7***
0wk	***Alginate bead-BMSC complex insertion surgery into subcutaneous layers of DVA/1J mice (4 weeks old)***					
2wk	***Histology*** (H & E, STRO for detection of human origin cell, Collagen type II, Aggrecan, Von kossa stain) ***RT-PCR*** (HIF-1α, GLUT-1 and Aggrecan)					
4wk	***Histology*** (H & E, STRO for detection of human origin cell, Collagen type II, Aggrecan) ***RT-PCR*** (HIF-1α, GLUT-1 and Aggrecan)					

A. RT-PCR at 2 and 4 weeks after implantation

In HIF-1α mRNA expression at 2 weeks, all groups demonstrated increased expression of HIF-1α mRNA compared to that of BMSC. BMSC with Ad-HIF-1α and Ad-GLUT-1, BMSC with Ad-GLUT-1, NP cell, BMSC with Ad-mock and BMSC with Ad-HIF-1α groups showed 17 fold, 4 fold, 3 fold, 3 fold and 2 fold increased level of HIF-1α mRNA expression compared to that of BMSC.

In GLUT-1 mRNA expression at 2 weeks, BMSC with Ad-HIF-1α and Ad-GLUT-1 showed 75% increased level of GLUT-1 mRNA expression compared to that of BMSC. BMSC with Ad-HIF-1α, NP cell and BMSC with Ad-GLUT-1 demonstrated comparable level of GLUT-1 mRNA expression with that of BMSC. But, BMSC with Ad-mock group showed 40% decrease in the GLUT-1 mRNA expression. (Figure 16)


BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
HIF-1α
GLUT-1
2weeks
β-actin


HIF-1α
Relative expression of mRNA
18
15
12
9
6
3
0
BMSC + Ad-mock
NP cell
BMSC + Ad-HIF-1α
BMSC + Ad-GLUT-1
BMSC + Ad-HIF-1α + Ad-GLUT-1

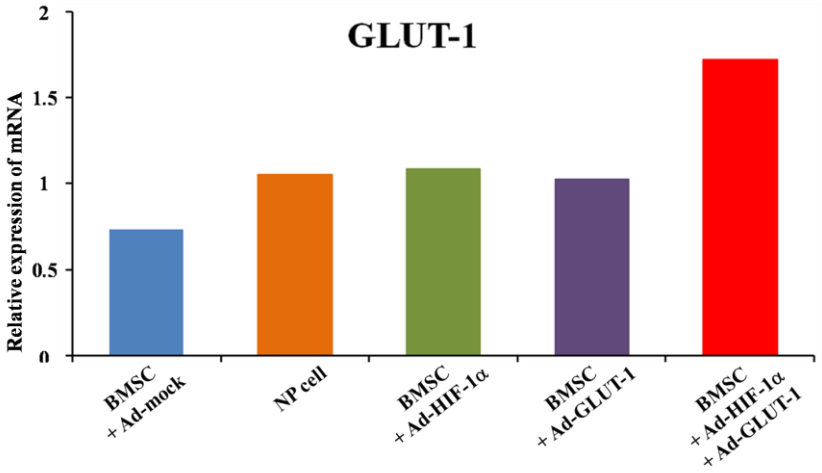


Figure 16. RT-PCR result of HIF-1α and GLUT-1 at 2 weeks after subcutaneous implantation in DVA/1J mice.

In HIF-1α mRNA expression at 2 weeks, all groups showed increased expression of HIF-1α mRNA compared to that of BMSC. In GLUT-1 mRNA expression, BMSC with Ad-HIF-1α and Ad-GLUT-1 revealed 75% increased level of GLUT-1 mRNA expression compared to that of BMSC. But, BMSC with Ad-mock group showed 40% decrease in the GLUT-1 mRNA expression.

In aggrecan mRNA expression at 2 weeks, all groups revealed increased expression of aggrecan mRNA compared to that of BMSC. BMSC with Ad-HIF-1α and Ad-GLUT-1, BMSC with Ad-GLUT-1, NP cell, BMSC with Ad-HIF-1α and BMSC with Ad-mock groups showed 250%, 70%, 60%, 20% and 10% increased level of aggrecan mRNA expression compared to that of BMSC. (Figure 17)

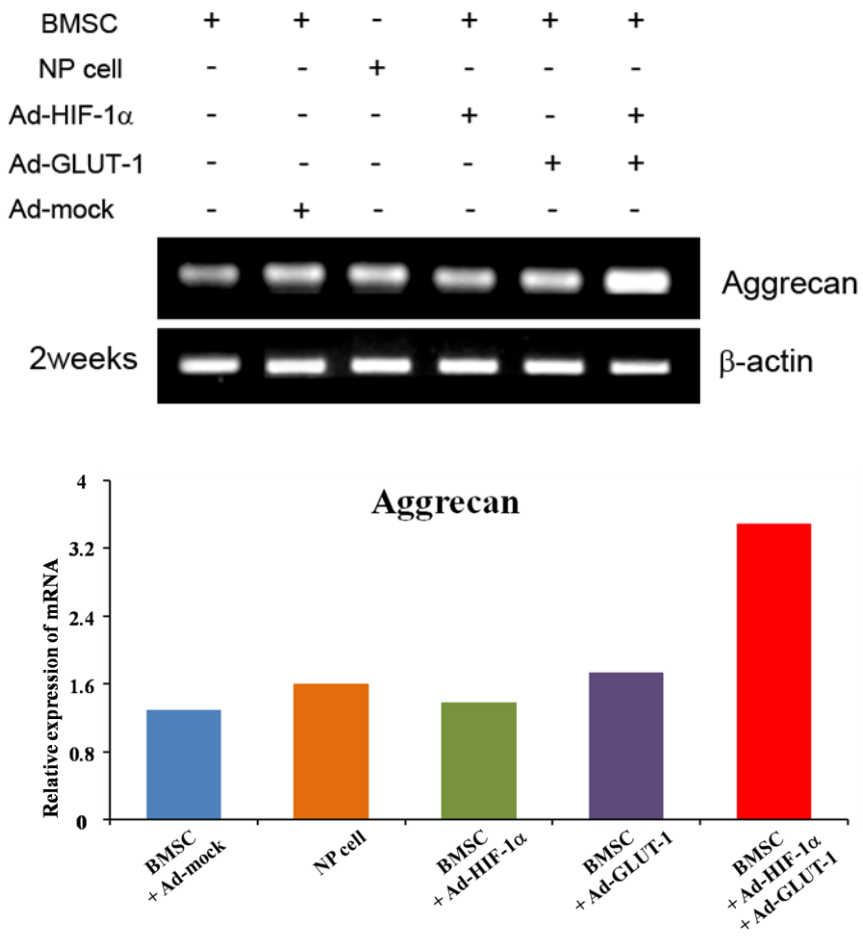


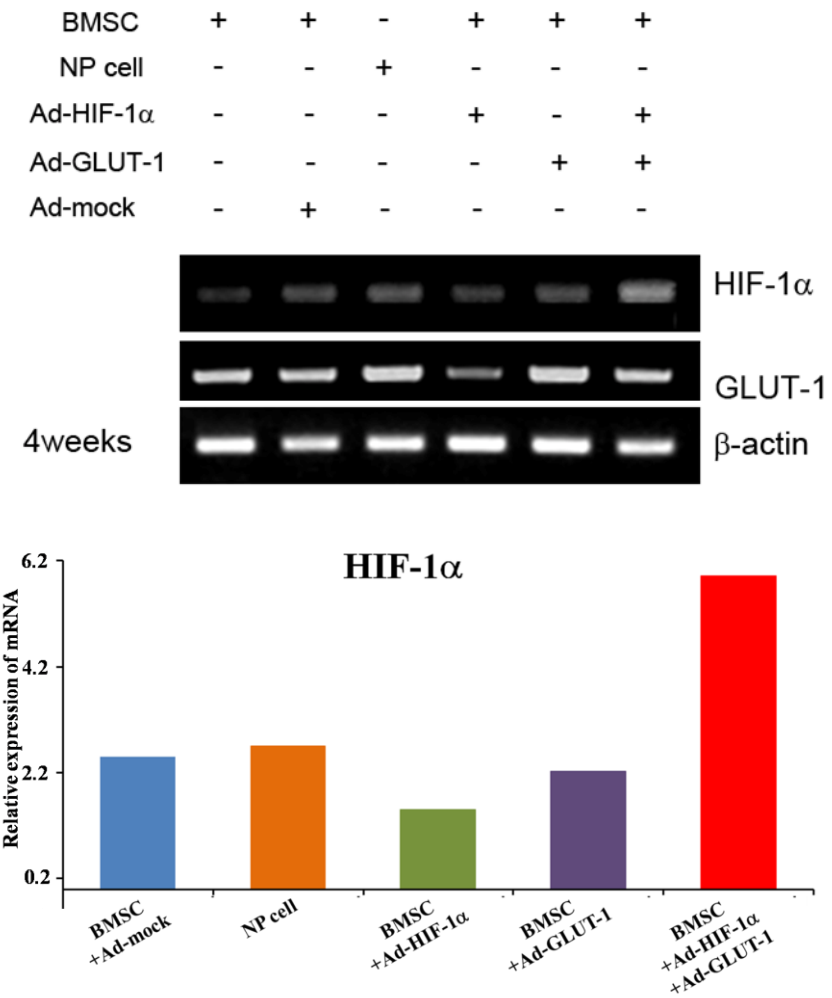
Figure 17. RT-PCR result of aggrecan at 2 weeks after subcutaneous implantation in DVA/1J mice.

In aggrecan mRNA expression, all groups showed increased expression of aggrecan mRNA compared to that of BMSC. BMSC with Ad-HIF-1α and Ad-GLUT-1 showed 250% increased level of aggrecan mRNA expression compared to that of BMSC.

In vivo result at 4 weeks, all measured value was inconsistent with other in vivo and in vitro results.

In HIF-1α mRNA expression at 4 weeks, all groups except BMSC with HIF-1α demonstrated increased expression of HIF-1α mRNA compared to that of BMSC. BMSC with Ad-HIF-1α and Ad-GLUT-1 group demonstrated 6 fold increased level of HIF-1α mRNA compared to that of BMSC group. BMSC with Ad-GLUT-1, NP cell, and BMSC with Ad-mock and BMSC with Ad-HIF-1α demonstrated 120%, 140% and 130% and 20% increased level of HIF-1α mRNA expression compared to that of BMSC.

In GLUT-1 mRNA expression at 4 weeks, all groups except BMSC with HIF-1α revealed increased expression of GLUT-1 mRNA compared to that of BMSC. BMSC with Ad-GLUT-1, NP cell, BMSC with Ad-HIF-1α and Ad-GLUT-1 and BMSC with Ad-mock showed 40%, 40%, 18% and 24% increased level of GLUT-1 mRNA expression compared to that of BMSC. BMSC with Ad-HIF-1α demonstrated 60% decreased level of GLUT-1 mRNA expression. (Figure 18)


BMSC + + - + + +
NP cell - - + - - -
Ad-HIF-1α - - - + - +
Ad-GLUT-1 - - - - + +
Ad-mock - + - - - -
HIF-1α
GLUT-1
β-actin
4weeks
HIF-1α
Relative expression of mRNA
6.2
4.2
2.2
0.2
BMSC +Ad-mock
NP cell
BMSC +Ad-HIF-1α
BMSC +Ad-GLUT-1
BMSC +Ad-HIF-1α +Ad-GLUT-1

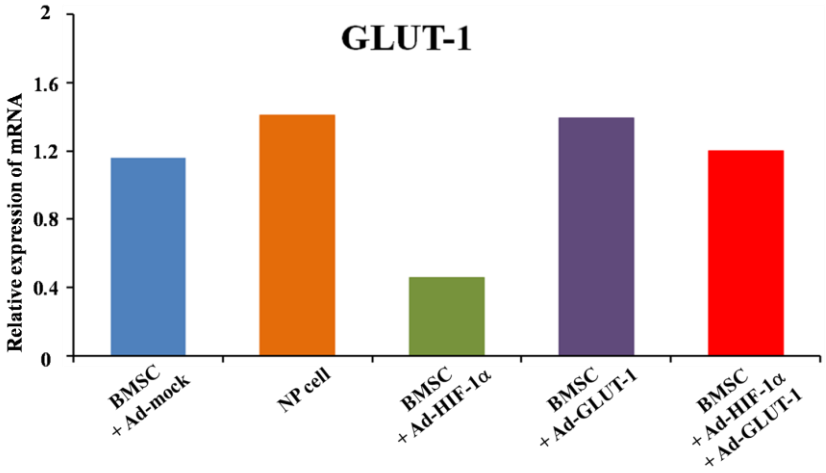


Figure 18. RT-PCR result of HIF-1α and GLUT-1 at 4 weeks after subcutaneous implantation in DVA/1J mice.

All data showed inconsistent level of mRNA expression in HIF-1α and GLUT-1 compared with other in vivo result at 2 weeks and in vitro results. In HIF-1α mRNA expression, all groups revealed increased expression of HIF-1α mRNA compared to that of BMSC. In GLUT-1 mRNA expression, all groups except BMSC with HIF-1α showed increased expression of GLUT-1 mRNA compared to that of BMSC.

In aggrecan mRNA expression at 4 weeks, all groups except BMSC with HIF-1α showed increased expression of aggrecan mRNA compared to that of BMSC. NP cell, BMSC with Ad-GLUT-1, BMSC with Ad-mock and BMSC with Ad-HIF-1α and Ad-GLUT-1 groups showed 200%, 120%, 70% and 60% increased level of aggrecan mRNA expression compared to that of BMSC. (Figure 19)

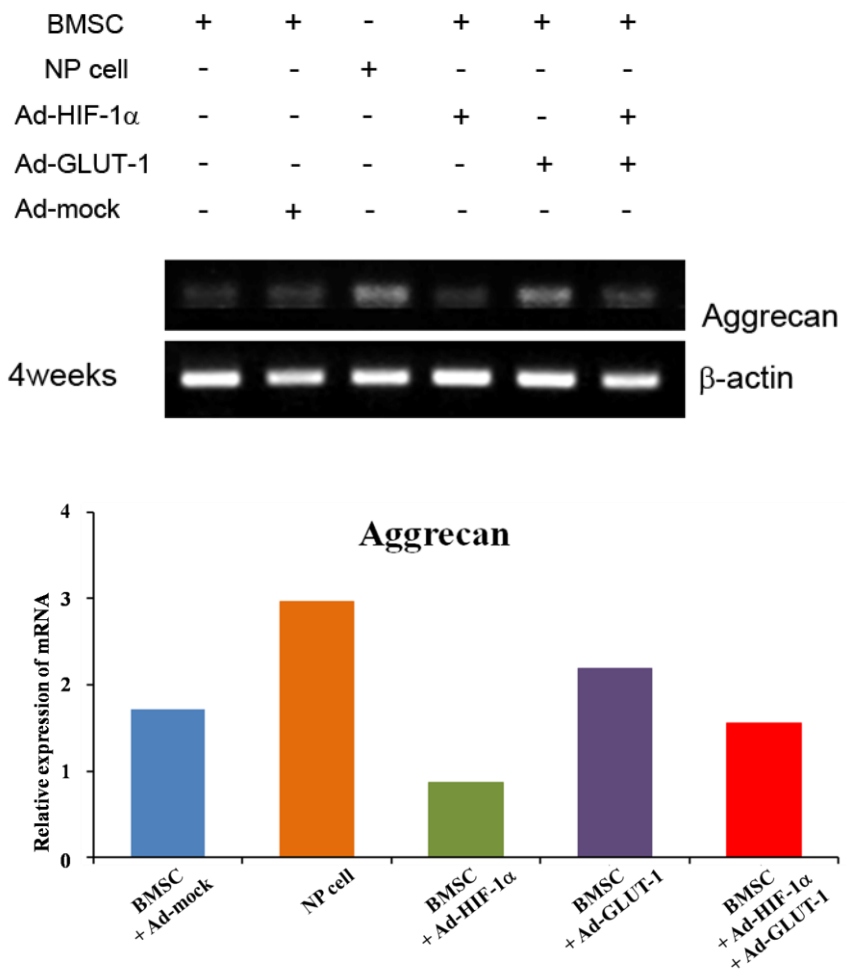


Figure 19. RT-PCR result of aggrecan at 4 weeks after subcutaneous implantation in DVA/1J mice.

Data demonstrated inconsistent level of mRNA expression in aggrecan compared with other in vivo result at 2 weeks and in vitro results. In aggrecan mRNA expression, all groups except BMSC with HIF-1α showed increased expression of aggrecan mRNA compared to that of BMSC.

B. Hematoxyline and Esosin stain

All groups demonstrated invagination of cells between alginate structures with stain. No vascular ingrowth with BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group due to Ad-HIF-1α gene transduction . (Figure 20)

In specimen at 2 weeks, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed larger cluster of cells between alginate components which were different from those of other groups.

In specimen at 4 weeks, heterogeneous types of cell population with different cell sizes and small crushed alginate components were observed as the mice-origin cells migrated and grew.

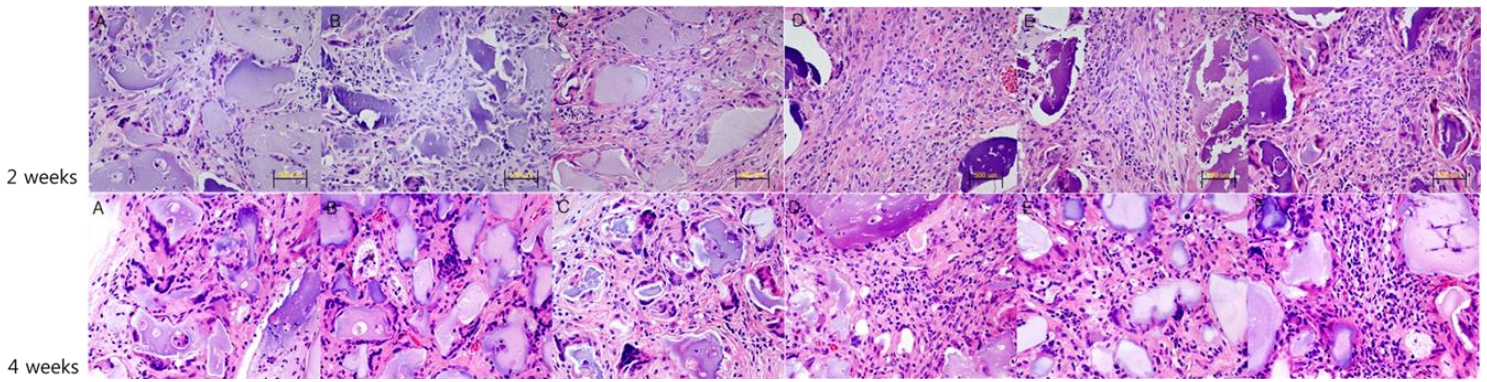


Figure 20. Hematoxylin and Esosin stain of cells in the alginate beads after 2 weeks and 4 weeks.(x40)

At 2 weeks, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group demonstrated larger cluster of cells between alginate components which were different from those of other groups compared to the other group. At 4 weeks, All group showed cells with different sized nuclei and cytosol which means heterogeneous cell migration from the host mice.

No vascular ingrowth with BMSC with Ad-HIF-1α and BMSC with Ad-HIF-1α and Ad-GLUT-1 group due to Ad-HIF-1α gene transduction .

A , BMSC; B, BMSC with Ad-mock; C, NP cell; D, BMSC with Ad-HIF-1α;E, BMSC with Ad-GLUT-1 ;

F, BMSC with Ad-HIF-1α and Ad-GLUT-1

C. STRO stain

All groups showed positive result for human mesenchymal stem cells, which means the retention and survival of human cells in the alginate beads. Cells with negative stain result were considered to come from the mice.

Although alginate components were degraded along with local immune reaction, the human origin cells were retained and presented as dark brown stained cells.

Until 4 weeks, human originated cells were detected in the alginate-BMSCs complexes. (Figure 21)

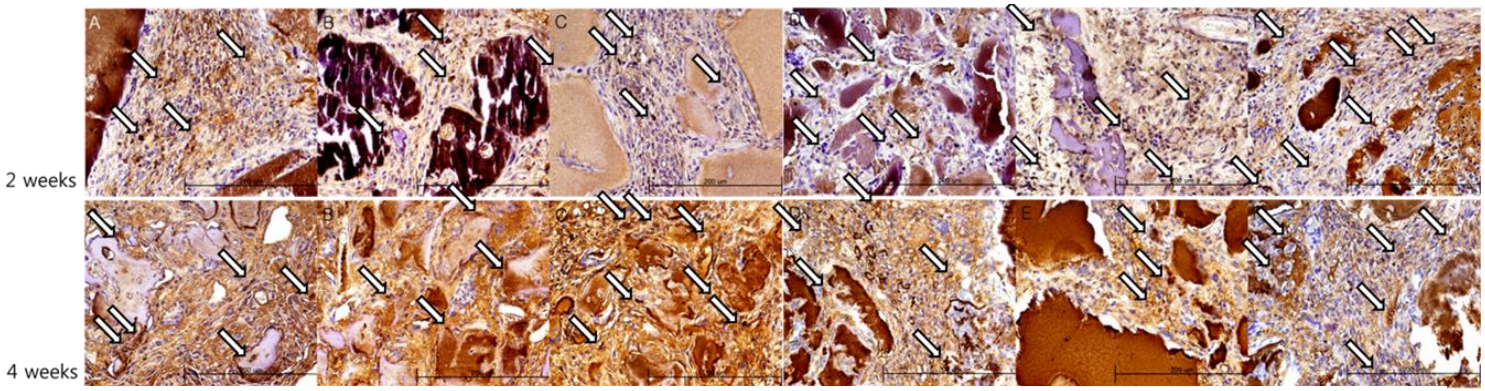


Figure 21. STRO stain for human origin bone marrow mesenchymal stem cell (BMSC) in the alginate beads after 2 weeks and 4 weeks (x40).

,All group showed positive results of dark brown stained cells indicated by arrows which were originated from the human BMSC at 2 weeks and 4 weeks.

Until 4 weeks after xenograft human BMSC into mice using alginate beads, human originated cells were retained in the alginate beads in all groups even local immune reaction was activated along with migrations of mice-originated cells.

A , BMSC; B, BMSC with Ad-mock; C, NP cell; D, BMSC with Ad-HIF-1α;E, BMSC with Ad-GLUT-1 ; F, BMSC with Ad-HIF-1α and Ad-GLUT-1

D. Collagen type II stain

In collagen type II stain in 2 weeks specimen, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of dark brown stained cells analogous to NP cell group which reflected the presence of collagen type II from differentiation of BMSC into NP cell.

In collagen type II stain in 4 weeks specimen, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group also showed positive result of dark brown stained cells analogous to NP cell group which meant the retention of collagen type II from the differentiated NP cell. (Figure 22)

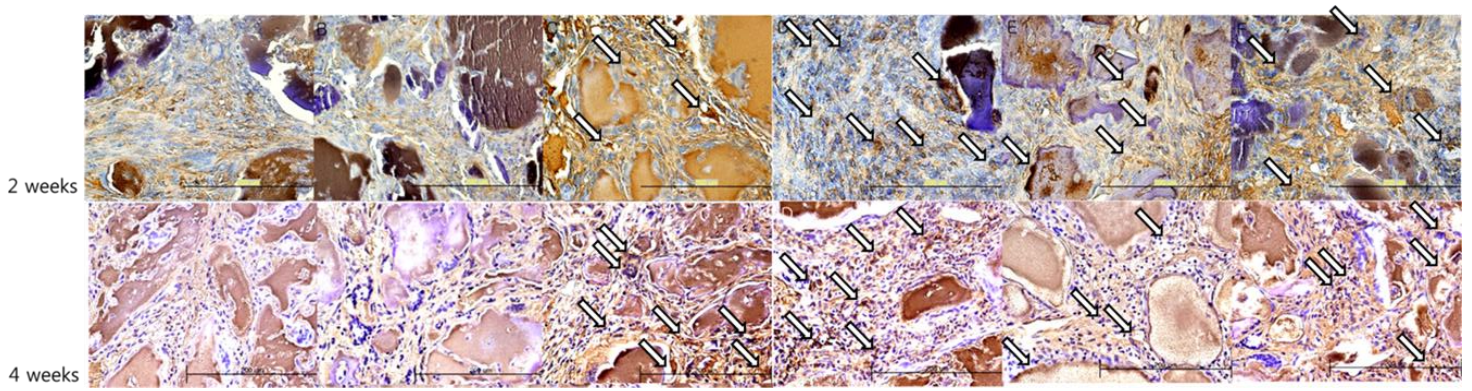


Figure 22. Collagen type II stain of cells in the alginate beads after 2 weeks and 4 weeks.(x40)

At 2 weeks, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of dark brown stained cells analogous to NP cell group, indicated by arrows, which reflected the presence of collagen type II from differentiation of BMSC into NP cell.

Until 4 weeks, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group still showed dark brown stained cells analogous to the NP cell group which were also indicated by arrows.

A , BMSC; B, BMSC with Ad-mock; C, NP cell; D, BMSC with Ad-HIF-1α; E, BMSC with Ad-GLUT-1 ; F, BMSC with Ad-HIF-1α and Ad-GLUT-1

E. Aggrecan stain

In aggrecan stain in 2 weeks specimen, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of dark brown stained cells analogous to NP cell group which reflected the presence of aggrecan from differentiation of BMSC into NP cell.

In the result of aggrecan stain in 4 weeks specimen, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group also showed positive result of dark brown stained cells analogous to NP cell group, which meant the retention of aggrecan from differentiated NP cell. (Figure 23)

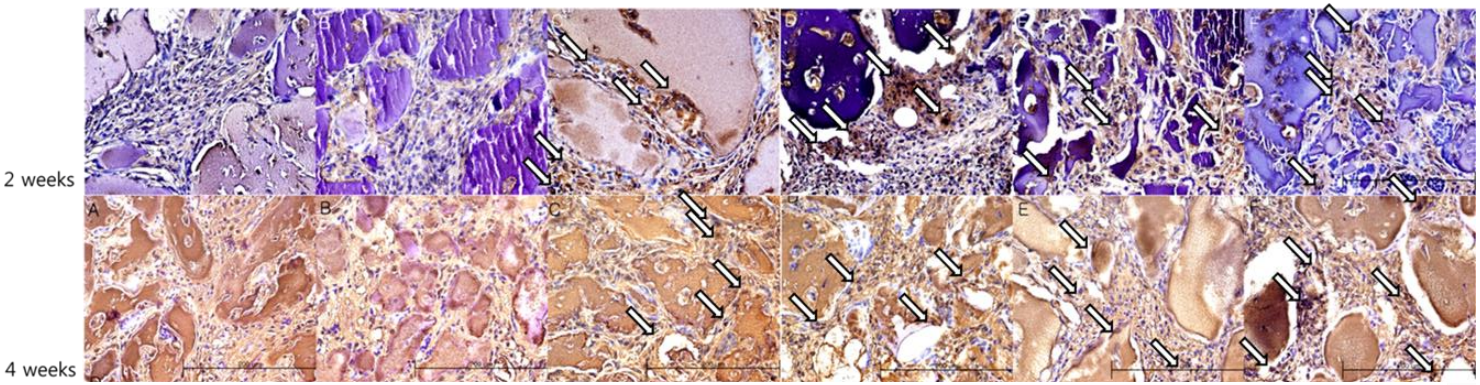


Figure 23. Aggrecan stain of cells in the alginate beads after 2 weeks and 4 weeks(x40). At 2 weeks, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group showed positive result of dark brown stained cells analogous to NP cell group, indicated by arrows, which reflected the presence of aggrecan from differentiation of BMSC into NP cell. Untile 4 weeks, BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1 and BMSC with Ad-HIF-1α and Ad-GLUT-1 group also showed positive result of dark brown stained cells analogous to the NP cell group, which meant the retention of aggrecan from differentiated NP cell.

A , BMSC; B, BMSC with Ad-mock; C, NP cell; D, BMSC with Ad-HIF-1α;

E, BMSC with Ad-GLUT-1 ; F, BMSC with Ad-HIF-1α and Ad-GLUT-1

F. Von kossa

All groups showed negative results for the detection of mineralization and calcification in the cytosol of differentiated cells and NP cell at 2weeks, which meant that no osteogenic differentiation of BMSC and NP cell by Ad-HIF-1α and Ad-GLUT-1 was progressed. (Figure 24.)

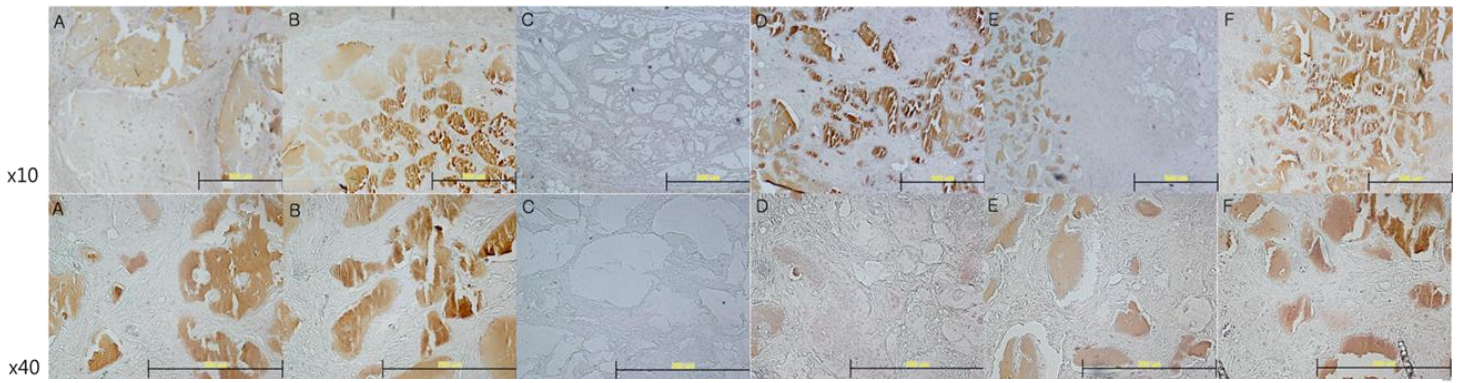


Figure 24. Von Kossa stain of cells in the alginate beads after 2 weeks (x10). Only interdigitated alginate was stained with brown color. All groups showed negative results for the detection of osteogenic differentiation of BMSC and NP cell.

A , BMSC; B, BMSC with Ad-mock; C, NP cell; D, BMSC with Ad-HIF-1α; E, BMSC with Ad-GLUT-1 ; F, BMSC with Ad-HIF-1α and Ad-GLUT-1

IV. DISCUSSION

The hallmark of disc degeneration is the progressive loss of proteoglycan, which coincides with decreases in oxygen tension, free radical accumulation, decreased pH, and the increased activity of aberrant proteolytic enzyme.[22-24]

Cell transplantation is one of the new therapy for degeneration of IVD that is based on the supplementation and/or replenishment of matrix-producing cells.[16] Among the candidates including annulus fibrosus(AF) cell,[25] elastic cartilage derived chondrocytes,[26] autologus or allogenous NP,[27,28] immortalized human NP cell line,[29, 30] mesenchymal stem cells (MSCs),[11, 15, 31] and embryogenic stem cells,[32] MSCs have some merits including availability from many autologuos sources without significant donor site morbidity,[16, 33] expandability in culture to produce adequate numbers of cells for transplantation,[15, 34] the preconditioning use of gene therapy to guide and stimulate MSCs toward the desired phenotype,[15, 16, 33, 35, 36] better potentiality to survive and produce significant quantitied of matrix,[16, 36] and immunologic privilege which enable the potential application of allogeneic MSCs as a therapeutic cell source.[37]

Still, researchers have not yet defined the NP cell by listing the specific gene expression profiles which describe NP cells. Instead, 'chondrocyte-like cell' is commonly used to call those specific cells. Confirmation of the characteristic markers of collagen type II, aggrecan and Sox-9 and the existence of HIF-1a can be used to distinguish NP cells from chondrocytes.[38]

Some in vitro studies have been successfully performed to differentiate BMSC into NP cell using a co-culture system,[11, 25, 34, 35, 39, 40] a cell carrier,[41] and a commercial scaffold.[42]

Also, various results as to enhancement of paracrine effect,[43] increase in revascularization[44] and prolongation of stem cell survival[45] were reported by preconditioning of MSCs via hypoxic condition[43, 45] and chemical conditioned media.[44]

Advances in stem cell biology have shown that differentiation of various MSCs depends primarily on the environment in which they are placed, suggesting hypoxic condition analogous to the unique original NP condition.[38, 46, 47]

Based on preconditioning gene transduction of BMSC on cell therapy,

phenotypic specific HIF-1 α and GLUT-1 were added in those cells to induce the NP specific differentiation in the present study. Additionally, stem cell differentiation after transduction of genes in normoxic condition, hypoxic condition and secondary hypoxic condition after normoxic preconditioning were compared in this study.

HIF-1 is a heterodimeric transcription factor composed of a stable and constitutively expressed β-subunit and oxygen-sensitive α-subunit.[48] The regulation of HIF-1α stability and degradation at the post-translational level under oxygen starvation has been investigated in numerous studies.[48-50] HIF-1 plays a major role in the pathobiology of other hypoxic condition such as ischemic heart disease, cancer, chronic lung disease and stroke.[51] HIF-1 mediated pathways influence angiogenesis, metabolic adaptation, innate immune response as well as survival and apoptosis of cells, and thus are critical factors in development, physiology and emergence of diseases like cancer and ischemia.[48] Under condition of reduced oxygen availability, HIF-1 regulates the expression of genes that mediate adaptive responses.[52, 53] HIF-1 was first identified in human cells as a regulator of erythropoietin, the

hormone that controls red-cell production by VEGF which stimulates angiogenesis and glycolytic enzyme which adapt cell metabolism to hypoxic condition.[51] HIF-1 is present in all nucleated cells of all metazoan species and plays an important role in both erythropoiesis and vascularization. [54]

HIF-1α serves as a key transcriptional factor that regulates the expression of enzymes concerned with glycolysis, the activity of the TCA cycle and oxidative phosphorylation,[55, 56] which mean adaptive responses to low oxygen tensions and nutrients deprivation.[57]

Maintenance of HIF-1α showed differentiation of BMSC into the NP-like phenotype and increase of the anti-apoptosis ability in the rabbit model.[58] Not only in the hypoxic condition but also in normoxic condition, HIF-1α serves to suppress oxidative phosphorylation, lower mitochondrial ATP generation, and promote glycolytic ATP and aggrecan synthesis.[59] These activities are of considerable functional importance and point to the role of HIF-1α in promoting the synthesis of key water-binding molecules of the extracellular matrix, complexes that are required to maintain the biomechanical properties of the IVD.[59]

The half-life of the HIF-1α protein is extremely short in normoxic condition and is markedly prolonged during hypoxic stimulation.[60] The mechanisms responsible for the rapid degradation of the HIF-1 α protein under normoxic condition were related to post-translational level modification of the α subunit such as ubiquitinization, acetylation, and phosphorylation,which influence protein stability and trans-activational function of HIF-1.[27, 48, 50, 61, 62] Recently, additional several pre-translational regulative mechanisms of HIF-1 has reported including transcriptional and post-transcriptional modifications. In accordance with post-translational regulation, again the modification of the α subunit plays the major role.[48]

In the present study, regardless of those degradative mechanism, the HIF-1α could be reproduced and remained to be effectively high level in the differentiated NP cell even in normoxic level, enough both to work as a phenotype specific differentiation introducer and to provide the syntechnic environment of BMSC differentiating into NP cell by gene therapy at once.

Additionally, the mRNA expression patterns of marix protein and transducted gene were different depending on the oxygen condition. It means

that the preconditioning in the normoxic condition renders not only to induce the BMSC to differentiate into the NP cell but also to facilitate the synthesis of matrix protein among three different oxygen conditions. Also, theoretically, normoxic preconditioning could upregulate the expression of transducted HIF-1α gene and the hypoxic metabolic pathways of the differentiated cells enough to be easily adapted in hypoxic condition even prior to expose them in hypoxic condition .

Therefore, the cell metabolism and possibly survival of induced NP cell could be affected favorably when the differentiation from BMSC into NP cell by the trasnducted HIF-1α was initiated, especially when BMSC with specific genes are placed in the preconditioning normoxic status and secondary hypoxic status.

Based on these merits, the pre-requisites of tissue engineering and regeneration could be gratified with the originality and reinforced durability of differentiated NP cell. Also, distinguished from the secondary increase of HIF-1 α in hypoxic condition,[38] the direct expression of transducted HIF-1α gene in the normoxic condition was confirmed to function properly in the

present study, which supports functionality of HIF-1α in NP under normoxic condition[48, 59] Until now, the regulation of HIF-1α under normoxia is essential interest regarding its distribution and regulation in cancer cells and only a few publications about non-hypoxic condition have been published.[27]

As mentioned to be the NP cell-markers in the previous studies,[34, 63, 64] collagen type II and aggrecan were increased as a result of transducted HIF-1α and GLUT-1 expression. Furthermore, based on the histological staining including hematoxyline and eosin, alcian blue, and Von kossa, the regulatory function of mineralization by HIF-1 in the IVD was detected which is correlating with recent studies.[65, 66]

Glucose transport and metabolism are hypoxia regulated processes in the avascular regions of the human IVD and that degeneration affects the expression of glucose transporters.[57] The result confirmed HIF-1α , GLUT-1,3,9 mRNA expression and co-expression of each GLUT isoform with HIF-1α in the NP. Also, immunochemistry demonstrated regional differences in GLUT expression, with the highest expression being in the NP. Regional differences in GLUT protein distribution was demonstrated reflecting hypoxic

status between NP and AF.[57] The result show that HIF-1α mRNA and the hypoxia responsive GLUT-1 genes are co-expressed in normal human IVD and that in the NP an increase in HIF-1α mRNA was associated with an increase in both GLUT-1 mRNA expression.[57]

It is reasonable to suggest that up-regulation of GLUT-1 in early degeneration may be a metabolic adaptation to the hypoxic condition, because the uptake of glucose by disc cell is a fundamental process.[57] One of the consequences of low oxygen tension in the NP is the reliance on glycolysis for energy generation,[59] known as one way in which HIF-1α promotes cell survival under hypoxic conditions is by mediating a switch from oxidative to glycolytic metabolism.[51] Glucose was converted to pyruvate by the glycolytic enzyme, which can be converted either to acetyl coenzyme A (CoA) for oxidation in the tricarboxylic acid cycle or to latate as a glycolytic end product.[8]

Immunochemical data from that study, in the NP, most hypoxic region of the IVD, the number of cells immunopositive for the GLUT-1, the most ubiquitous of the GLUTs, this increase continue to late stage of degeneration, which considered to be a response to the lack of available glucose within the

extracellular matrix.[57]

As a result of our investigation on HIF-1α and GLUT-1, GLUT-1 alone could induce the synthesis of NP specific matrix proteins in a certain amount.

According to the well-known sequence between HIF-1α and GLUT-1,[57] HIF-1α is not induced by GLUT-1 in the present study. Therefore, the commonly accepted concept on NP cell, which explains itself as a chondrocyte like cell with a necessary phenotypic marker of HIF-1α and NP specific matrix protiens such as collagen type II, aggrecan and SOX-9,[38] could not be satisfied with transduction of GLUT-1 alone in normoxic condition. Another preconditioning in the hypoxic condition might be necessary to induce BMSC into NP cell with transduction of GLUT-1 gene alone. Nevertheless, transduction of GLUT-1 as a phenotypic marker of NP cell showed considerable effect on synthesis of NP matrix in the present study. Also, transducted GLUT-1 gene in combination with Ad-HIF-1α showed some additional effects in the synthesis of NP specific matrix proteins in a certain circumstance such as the result of in vivo 2 weeks.

It is a common phenomenon that the expression of HIF-1α was reduced when

the GLUT-1 gene had transducted alone or in combination with Ad-HIF-1 α regardless of oxygen status at 96 hour in vitro culture, discerning from the 48 hour in vitro result. It might be derived from the sequential relation between HIF-1α and GLUT-1,[57] and possible negative feedback by GLUT-1 on HIF-1α expression could be postulated and further study on this mention is strongly necessitated.

Although the present study could be considered as the highlighting study for the potential for BMSC therapy in degenerative IVD using NP specific HIF-1 α and GLUT-1 via adenoviral vector, many questions are still remained to be solved. The estimated survival after transplantation into the human degenerative IVD, extensibility of the survival of grafts in combination with the growth factor or blocking specific enzyme gene such as BMP-2 and TIMP[67] should be investigated. But most of all, the safety control the viral vector related risk during the human trial should be answered.[68]

As for containing scaffold, alginate beads scaffold was used during differentiation of the BMSC into NP cell which proved to be effective and safe to maintain the viability of human BMSC.[69] Considering the

characteristic of alginate bead in differentiation of BMSC into NP cell, environmental comparability could be induced compared with natural course of the embryogenic differentiation and degeneration of NP. Because, the alginate beads are three dimensional tissue engineering constructs, nutrients and oxygen are replenished by diffusion.[69] Therefore, a nutrient gradient developes where cells on the exterior portions of scaffolds receive sufficient nutrients, whereas cells on the interior of scaffolds are deprived of nutrients and are exposed to hypoxic condition.[69] Aggregated alginate constructs were proved to be of sufficient strength for the engineering of tissue such as cartilage,[70, 71] non-load bearing bone,[72] and skelektal muscle.[73] As a result of in vivo experiment, spherical alginate beads were deformed into long oval sphere due to subcutaneous pressure of mice, which resulted in the only thin layers of enveloped cells clusters left. Therefore, an additional mechanical support of alginate beads or pressure-free space to encourage the cell differentiation and volumetric growth is required.

In summary, an effort to differentiate the BMSC into the designated NP cell by transducted HIF-1 and GLUT-1 in vivo and in vitro was successful. For the

future study, the survival and cell metabolic change related to the preconditioning oxygen status and three dimensional growth should be investigated thoroughly.

V. CONCLUSION

Transduction of each gene of the HIF-1α and GLUT-1 by adenovirus proved to be effective to induce the differentiation of BMSC into NP cell in vitro and in vivo. The phenotypic expression and matrix synthesis depending on the combination of the transducted genes in company with the oxygen preconditioning were consistent and expectable. Thus this result show us new suggestions in the treatment of degenerative IVD in the way of differentiation of new NP-like cell from BMSC and possible modulation of the cell metabolism based on transduction of HIF-1α and GLUT-1 genes.

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ABSTRACT(IN KOREAN)

Discogenic differentiation of human bone marrow mesenchymal stem cell with Adenovirus mediated glucose transporter-1 and hypoxia inducible factor-1α gene therapy

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연령이 증가하고 퇴행성 변화가 진행됨에 따라 디스크는 점차 기질적인 변화와 형태학적인 변화 그리고, 세포 수준의 변화를 겪게 된다. 이런 디스크의 퇴행성 변화를 치료하는 이상적인 방법으로는 손상받은 디스크 조직을 원래의 디스크와 생물학적 및 생화학적으로 비슷하거나 더 나은 성질을 가지는 기질적 구조로 수리 및 대체하는 방법이 있다. 최근, 생물학적으로 손상된 조직을 수리하거나, 디스크의 기능을 재생하는 분야에 대한 연구가 유전자치료, 성장인자 주입, 세포 기반 조직 공학등의 분야에서 활발하게 이뤄지고 있다.

Hypoxia inducible factor-1α (이하 HIF-1α) 는 디스크 내에서 에너지 대사와 세포의 생존을 조절하는 중요한 인자이며, 또한 인간의 디스크 세포의 특징을 나타내는 표현형 인자로 밝혀진 바 있다. Glucose transporter-1(이하 GLUT-1)은 디스크의 특징적인 환경인 저산소 환경에서 디스크 세포내로의 glucose의 운반을 촉진하는 역할을 하며, 역시 HIF-1α와 마찬가지로 디스크 세포의 표현형 인자로 사용될 수 있다.

본 연구에서는 이런 디스크 세포에 특징적인 표현형 인자인 HIF-1α 와 GLUT-1을 아데노 바이러스를 이용한 골수 중간엽 줄기세포(BMSC)로의 유전자 전달을 통해 디스크 세포로의 분화 가능성에 대해 연구하고자 실험을 진행하였다.

BMSC 는 척추관 협착증 환자의 수술이 진행되는 동안 해당환자에게서 환자의 술전 동의 하에 채집되었다.

대조군과 실험군을 설정한 후 각각 시험관내 (in vitro) 와 생체내 (in vivo) 실험을 동시에 진행하였다. 시험관내 실험에서는 디스크 세포를 양성 대조군으로 설정하였고, BMSC를 음성 대조군으로 설정하여, 각각의 대조군과 주입된 유전자 등의 조합하여 총 6군에 대

하여 실험을 진행하였다

아데노바이러스를 이용한 유전자 전달 및 그에 따른 BMSC의 디스크 세포로의 분화는 각각 reverse-trasctiption polymerase chain reaction (RT-PCR) 과 조직학적 검사등을 통해 확인하였다.

생체내 실험에서는 alginate-BMSC 복합체를 mice(DVA/1J)의 배부에 피하 이종 이식을 시행 후, 각각 2 주 및 4 주에 희생시켜 조직을 채취 후 조직학적 검사를 시행하였다. 최종 산물에 대해 aggrecan과 collagen type II 존재를 확인하여 디스크 세포로의 분화를 검증하였다.

시험관내 실험의 각 24시간 및 48 시간 결과, BMSC with Ad-HIF-1α 와 BMSC with Ad-GLUT-1 그리고 BMSC with Ad-HIF-1α and Ad-GLUT-1 군에서 aggrecan과 GLUT-1 그리고 collagen type II가 디스크 세포와 비슷한 정도의 mRNA를 발현하는 것으로 확인되었다. 그러나 HIF-1α의 mRNA의 발현은 HIF-1α의 유전자를 주입한 BMSC with Ad-HIF-1α 와 BMSC with Ad-HIF-1α and Ad-GLUT-1 군에서만 증가하는 것으로 관찰되었다.

각각 96시간 동안 산소 농도를 다르게 하여 배양한 실험에서는,

48시간 저 산소 농도 배양 후 48시간 정상 산소농도에서 배양한 군에서는 정상산소 농도를 유지한 군에서 각각의 HIF-1α와 GLUT-1의 mRNA 발현은 96시간동안 정상 산소 농도를 유지한 군과 비슷하게 나타났다. 하지만 기질 합성에서는 aggrecan과 제2형 콜라겐의 mRNA발현이 96시간동안 저산소 농도 배양을 유지한 군과 비슷한 양상으로 발현되었다.

생체내 실험 결과로는, 제2형 콜라겐과 aggrecan에 대한 양성 염색 소견이 각각 BMSC with Ad-HIF-1α, BMSC with Ad-GLUT-1, BMSC with Ad-HIF-1α and Ad-GLUT-1 군에서 모두 디스크 세포 군과 비슷한 정도로 발현되었다.

따라서 본 연구에서의 HIF-1α와 GLUT-1 유전자의 아데노바이러스를 통한 BMSC로의 전달을 통한 디스크형 세포로의 분화 유도는 시험관내 와 생체내 실험 모두에서 성공적이었음에 대해 확인하였다.

핵심되는 말 : HIF-1α, GLUT-1, 골수 중간엽 줄기세포, 아데노바이러스, 디스크 세포